2010

Inhibition of Cell Invasion by Targeting PLD

Terry C. Farkaly
Wright State University

INHIBITION OF CELL INVASION BY TARGETING PLD

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

TERRY FARKALY
B.S., St. Joseph's College, 2008

2010
Wright State University

WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

June 24, 2010

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Terry Farkaly ENTITLED The Inhibition of Cell Invasion by Targeting Phospholipase D BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Julian Gomez-Cambronero, Ph.D.

Steven Berberich, Ph.D.
Department Chair

Committee on
Final Examination

Julian Gomez-Cambronero, Ph.D.

Michael Leffak, Ph.D.

Steven Berberich, Ph.D.

Jack Bantle, Ph.D.
Dean, School of Graduate Studies

ABSTRACT

Farkaly, Terry. M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2010. Inhibition of Cell Invasion by Targeting Phospholipase D.

Phospholipase D (PLD) is a crucial signaling enzyme involved in many cellular processes. The catalytic activity of PLD is essential for the production of Phosphatidic Acid (PA), a critical second messenger in cell signaling cascades downstream. Using the highly invasive rat mammary adenocarcinoma cell line mTLn3 as a metastatic model, we investigated the proficiency of these cells to invade using matrigels that mimic the basement membrane of the extracellular matrix (ECM), their activity through PLD enzymatic assays, as well as the potency of our potential inhibitors to inhibit PLD-mediated cell invasion and lipase activity.

This study reveals that PLD-mediated cell invasion is dependent on protein-protein interactions with other cell signaling molecules such as Grb2 and Rac2 and their effect on lipase activity. Regulation of PLD2 activity by phosphorylation on tyrosine residue sites within its Phox domain are examined; in which we elucidate the effects of specific kinases EGFR, Jak3 and Src on cell invasion and enzymatic activity. Based off this approach, a therapeutic resolution will be proposed to diminish cell invasion in metastasis.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figures Page

LIST OF TABLES

Table Page

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Julian Gomez-Cambronero for the oportunity to be a part of this lab and his guidance throughout this project. I thank Kathy Frondorf for her support and advice given to me throughout my stay at Wright State, without her I never would have been able to get started in this lab. I would also like to thank Karen Henkels, who was always there to answer the hundreds of questions that I had over the past year and who would continually go out of her way to support the writing of this thesis, even when she was on vacation. I also thank my committee members, Dr. Michael Leffak and Dr. Steven Berberich, their guidance throughout my time spent here is truly appreciated. Finally, I would like to thank my family for their never-ending support, especially Mom, without whom I wouldn't be here today.

I. INTRODUCTION

Cell Invasion in Metastasis

Metastasis is a multi-step model comprised of the growth of a primary tumor, intravasation (cell invasion), arrest and growth in a secondary site [1]. One such example is breast cancer, the 2nd leading cause of cancer death in Western nations (1st among women), the primary cause of mortality being the metastatic spread of tumor cells from their primary site to other organs in the body [2]. By limiting cell invasiveness, tumor cells would be unable to enter the bloodstream and extravasate to distant sites in the body, stopping the spread of cancer. The cell line chosen to study this metastatic model was the MTLn3 rat mammary adenocarcinoma, an optimal model to study breast cancer progression and treatment [3]. Increased tumor cell motility and invasion is often the consequence of an overexpressed epidermal growth factor receptor [4], in which we elucidated one of the cellular pathways affected here.

II. Literature Review

The Enzymatic Role of Phospholipase D

Phospholipase D (PLD) is an enzyme located within the plasma membrane that catalyzes the hydrolysis of phosphatidylcholine, targeting its ester linkage to yield the product phosphatidic acid (PA) and the release of a soluble choline headgroup. When water is replaced as the nucleophilic acceptor by a primary alcohol such as butanol, PLD can yield phosphatidylbutanol through a second reaction known as transphosphatidylation (Figure 1) [5,6]. This catalytic reaction allows us to assay an indirect measurement of PLD activity *in vitro* by quantifying the amount of [^{3}H]phosphatidyl-butanol produced within the cell from exogenous [^{3}H]butanol.

A Brief History and Background of Phospholipase D

The discovery of PLD in plants came in 1947 by Hanahan and Cahikoff, who were the first to describe its enzymatic activity in carrots [7,8]. In animals, PLD was found to suddenly activate in a direct response to extracellular stimuli [9]. PLD is also an important regulator of many cellular functions through the production of second messengers such as PA and the products of its metabolism, diacylglycerol (DAG) and lysophatidic acid (LPA), all of which are involved with numerous cellular and intracellular sigaling processes [9-11].

Mammalian PLD Structure

There are 2 isoforms of the PLD gene, PLD1 and PLD2 (Figure 2). Park et. al localized each gene through *in situ* hybridization, PLD1 in the long arm (q) of chromosome three (3q26) and PLD2 in the short arm (p) of chromosome seventeen

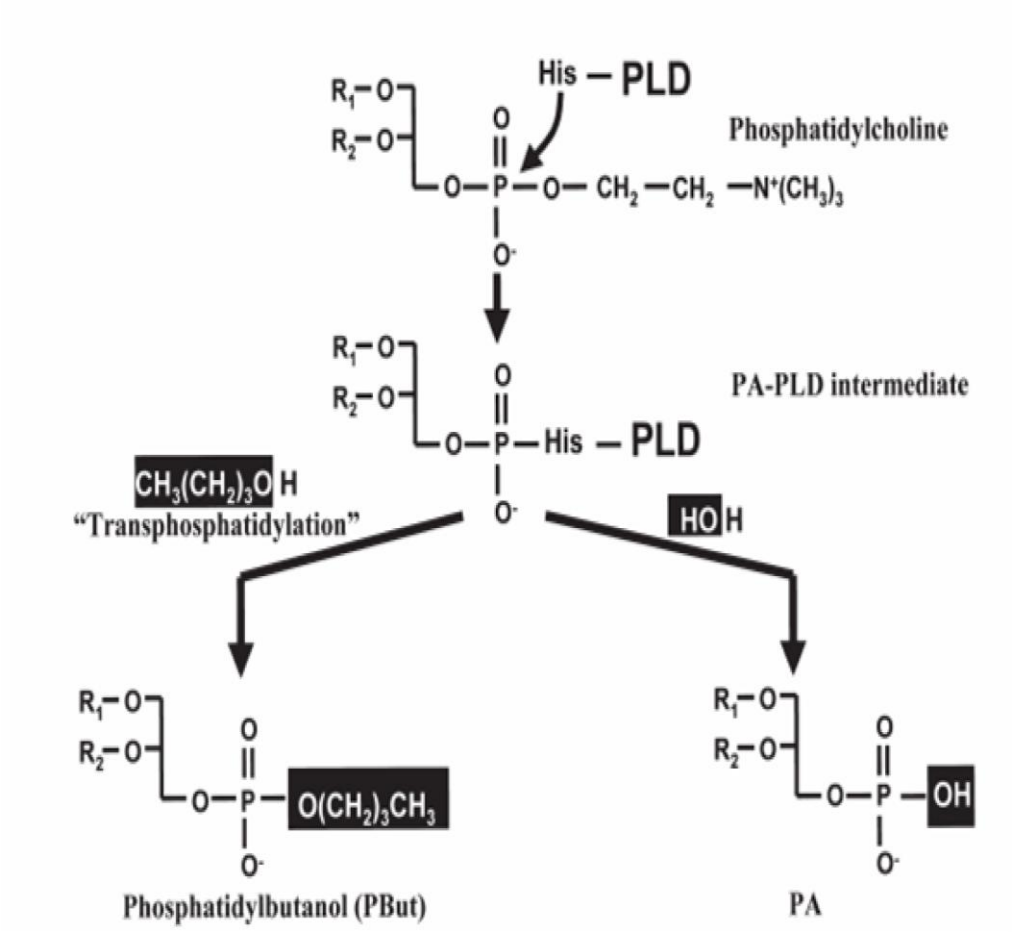


Figure 1: In the first part of the reaction, a PA-PLD intermediate is formed by a covalent linkage of PA to a histidine. In the second part of the reaction, two outcomes may occur. Hydrolysis will produce PA when H_2O is present; transphosphatidylation will produce a phosphatidylalcohol when a primary alcohol (such as butanol) is present [5].

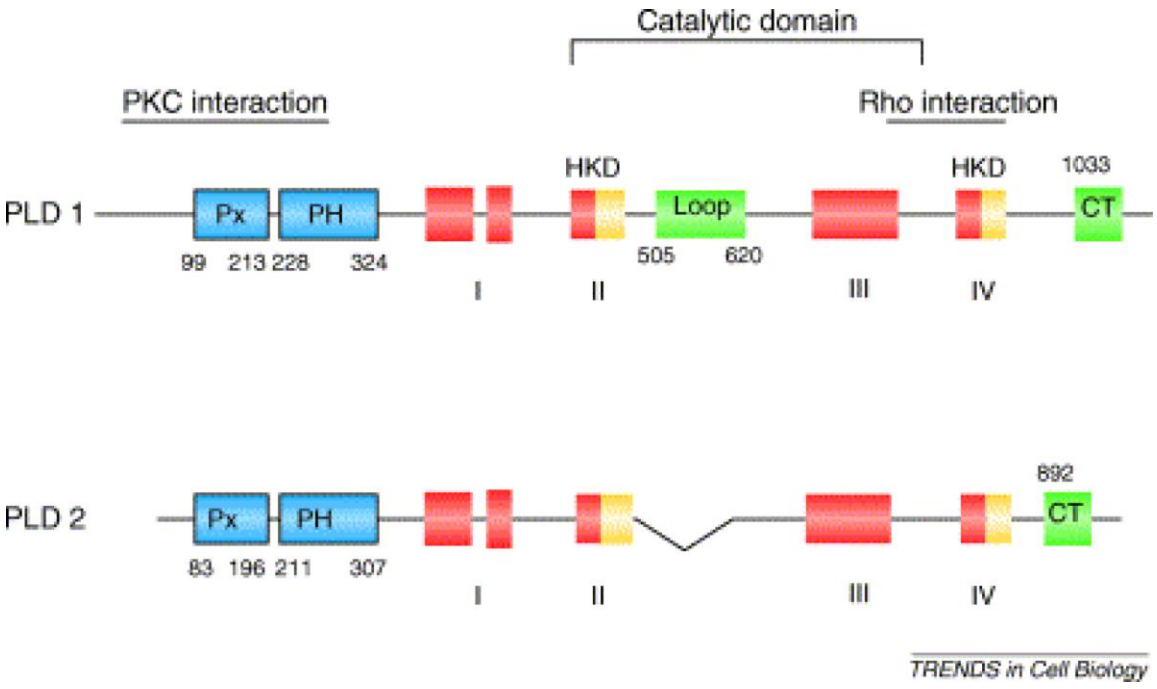


Figure 2: The four conserved regions are indicated by roman numerals, the 'HKD' motifs responsible for catalytic activity are in yellow. PLD1 possesses a 116-residue activation loop not found in PLD2. [12].

(17p13) [13,14]. Approximately 50% amino acid homology is shared between the two [15]. The PLD1 gene is a 120 kDa protein that encodes an 1074-amino acid protein; PLD2 encodes a 933-amino acid protein with a molecular weight of 106 kDa [9]. Four splice variants are yielded from PLD1 (PLD1a1, PLD1a2, PLD1b1, PLD1b2) [16]. PLD2 yields two splice variants, PLD2a and PLD2b, functionally indistinguishable from the other [17].

All members of the PLD superfamily contain two highly conserved catalytic domains known as HKD motifs ($HXKX_4DX_6$), essential for enzymatic activity [18]. Other conserved regions within the PLD family include a PI4,5P_2 (PIP_2) binding site, pleckstrin homology (PH) domain, and *phox* homology (PX) domain [19,20]. The PX domain, located at the N terminus, is involved in intracellular protein localization, enabling binding to SH2/SH3-containing tyrosine kinases [21]. The PX domain also serves as a binding motif for proteins or lipids and has been shown to function as a GTPase activating protein [22].

Tissue and Subcellular Distribution of PLD1 & PLD2

Steed et. al found that human PLD1 does not appear to be an abundant message in tissue whereas levels of human PLD2 mRNA were higher and more variable [17]. In cancers with elevated tyrosine kinase expression, such as breast cancer, elevated expression of PLD1 and elevated PLD activity was reported in breast cancer tissues [23,24]. Elevated PLD activity has also been observed in gastric [25] and renal cancers [26], and a polymorphism on the PLD2 gene has been associated with the prevalence of colorectal cancer [27].

Both PLD1 and PLD2 are palmitoylated on conserved Cys residues; this fatty acylation being a likely contributor to membrane association [28]. PLD1 is located throughout the cell, primarily in the perinuclear, Golgi and heavy membrane fractions [28].

Endogenous PLD2 is evident on the plasma membrane; however, much of the enzyme is localized to the Golgi region and cytosolic puncta [29]. Overexpressed PLD2 is primarily found on the plasma membrane, where association with ARF6 (small-GTPase that regulates the membrane trafficking between the plasma membrane and endosome)[30] and EGF receptor occurs at sites of membrane ruffling [31]. PLD2 is also present in the cytosol [31], as well as a submembraneous vesicle compartment likely endocytic in origin that translocates to the plasma membrane when stimulated by EGF [31].

Intracellular Regulators of PLD2

PLD1 exhibits low basal activity that requires activation by numerous factors, while PLD2 exhibits high basal activity [17]. Due to the difficulty of transfecting multiple PLD1 activators into the cell, we instead focus largely on PLD2 in this study.

PLD2 is subject to complex regulation by numerous molecules such as the Rac and Rho-GTPase family, protein kinase C (PKC) and alternative reading frame tumor suppressor (ARF) [5]. PLD2 can be modestly activated by ARF proteins [32]. Some positive response from PLD2 from phorbol esters has also been observed, indicating a role for PKCα in PLD2 regulation [33]. PLD2 is also activated by unsaturated fatty acids such as oleate, linoleate and arachidonate [34]. Additionally, upregulation of PLD2 occurs through cell surface receptors when cells are stimulated by EGF or insulin, PLD2 will become tyrosine-phosphorylated and form a complex with the EGF receptor [35]. PIP_2 is required for PLD activity [36]. Phosphoinositide regulation by PIP_2 has been identified to occur between motifs II and IV on PLD2, a conserved region of 10-20 residues known as 'KR' motifs **(18).** When mutated, PIP_2 dependent activation is lost [19]. PLD2 activity is also

regulated by phosphorylation-dephosphorylation by 3 specific kinases: EGF-R, Jak3 and Src, with negative, positive and ambivalent modulation, respectively [37].

Physiological Functions of PLD

PLD plays a role in numerous physiological and pathological processes, some of which include: endocytosis, exocytosis, membrane vesicle trafficking, cell migration, mitosis, cell survival, growth, transformation and tumor progression [38], actin cytoskeleton remodeling and stimulation of actin stress fiber formation [31,39], mitogenic signal transduction and oncogenesis [40,41], inflammation [42,43], phagocytosis [44] and leukocyte chemotaxis, cell polarity, adhesion and actin polymerization [45].

PLD-derived PA can function as a membrane anchor that transiently targets specific proteins to recruit or activate PA-domain containing proteins, such as the kinase Raf-1 [46,47]. The list of PA binding proteins is extensive, some of which include: PI4P5K (generates PIP_2 from PIP), mammalian target of rapamycin (mTOR), ribosomal p70 S6 kinase (p70S6K), small GTPase Rac, kinase suppressor of Ras and the Ras guanine nucleotide-exchange factor Son of *sevenless* (Sos) [48,49]. PIP2 synthesis by the PI4P5 kinase, a downstream target of PLD signaling, can be stimulated by PA [5], because PLD is activated by PIP2, this interaction may represent a positive feedback loop that activates PLD2 in lipid rafts [38]. PA will also function in cellular signaling pathways as a direct lipid second messenger or as an indirect precursor, once converted to other bioactive lipids such as LPA and DAG [9-11]. Generation of PA engineers the recruitment of Raf to the plasma membrane, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway [38,46,47]. PA is also required for activation of mTORC1 in mTOR, the

protein kinase that regulates both cell cycle progression and cell growth [50], both downstream targets of PLD implicated in cell survival. A downstream target of mTOR, p70S6K, is phosphorylated, catalyzing the phosphorylation of the S6 protein to initiate protein synthesis [51,52]. PA also activates S6K independently of mTOR [53]. Not only does PLD enhance cell proliferation and survival, it can also prevent cell cycle arrest [54] and apoptosis through survival signaling [55].

Grb2 and its Interaction with PLD2

Growth receptor bound protein 2 (Grb2) is a 25 kDa adapter protein comprised of one SH2 domain and two SH3 domains responsible for signal transduction between growth factor receptors and intracellular signaling cascades [56]. Grb2 is essential in cellular proliferation [57,58] and PLD2 lipase activity, intracellular localization and signaling in response to EGF [59]. In colorectal carcinoma, Grb2 expression and genes involved in Grb2-mediated pathways were highly elevated in the metastases due to its role in tumor growth, invasiveness and metastasis [60]. Cytoplasmic Grb2 relocates to the plasma membrane when stimulated by EGF and binds to the EGF receptor through phosphotyrosine containing proteins located on its SH2 domain [61]. The Grb2 SH2 domain, in addition, serves for recruitment to the phosphotyrosine motif *p-YxN* located on the PH domain of PLD2 [62]. The two SH3 domains of Grb2 interact with proline-rich motifs of signaling proteins, the most important being Sos [63]. The Grb2/Sos complex can form independently of PLD2 and has been associated with embryogenesis, regulation of the cytoskeleton, DNA synthesis and cancer [57,61,64]. Once the Grb2/Sos complex is formed, mitogenic Ras effectors are activated, stimulating cellular proliferation and the

MAPK cascade downstream [63]. PLD2 binds Grb2 and recruits Sos, while PLD-derived PA can bind Sos directly, both interactions are able to promote GTP loading of Ras and the stimulation of the MEK/ERK pathways [65]. PLD2 residues Y^{169} and Y^{179} are a necessary requirement to directly bind Grb2 and recruit Sos *in vivo*, Y^{169} modulates activity of the enzyme while Y^{179} regulates tyrosine phosphorylation of the protein [21]. In addition to these sites, Y^{511} has also been shown as a tyrosine interaction site between PLD2 and Grb2 [66]. When Y^{179} and Y^{511} are phosphorylated, PLD2/Grb2 mediates lipase activity, when dephosphorylated, lipase activity decreases and mediates an induction of cell proliferation and *de novo* DNA synthesis [66].

Rac2, CRIB, and their Interaction with PLD2

Rac2 is a GTPase belonging to the Ras superfamily of small GTP-binding proteins, involved in cell spreading, migration, mitogenesis, actin cytoskeletal rearrangement, phagocytosis, superoxide generation and axonal growth [67]. Innate immune responses depend upon neutrophil movement and phagoctyic leukocytes to respond to invading pathogens [68]. The first human disease discovered due to a mutation of a Rho GTPase was human neutrophil deficiency syndrome, due to an inhibitory Rac2 mutation, as Rac2 is a critical regulator of chemotaxis [68,69]. Rac2 is involved in regulation of hematopoietic stem cells (HSC), actin polymerization, adhesion and migration, phagocyte NADPH oxidase, and along with Rac1, the ability to individually control HSC cell cycle progression and survival [70,71]. Rho GTPases have a significant role in the development of cancer; these roles include cell transformation, proliferation, invasion, metastasis, and

angiogenesis, with recent research showing a remarkable increase of Rac2 expression in head and neck carcinoma [72,73].

Peng et. al has demonstrated for the first time that upon cell stimulation, PLD2 translocates from the nucleus and golgi region to the cell membrane where it interacts with Rac2 (unpublished results). Rac2 binds to the PH domain of PLD2 at a newly-identified Cdc42/Rac Interactive Binding (CRIB) domain and affects lipase activity. The protein interaction between PLD2 and Rac2 are shown in Figure 3.

A Rac2-PLD2 interaction could exist and serve to modulate PLD enzyme activity, perhaps leading to an enhanced functionality where both molecules have been implicated. When EGF is present, Henkels et. al have shown that Rac2 protein expression increases substantially when co-transfected with PLD2 (Figure 4). This increase in Rac2 protein expression indicates that PLD2, when stimulated by EGF, upregulates Rac2 protein expression.

MTLn3 Cell Line

MTLn3 cells are a highly invasive and metastatic rat mammary adenocarcinoma, a widely used model to observe metastases. MTLn3 cells overexpress EGF receptors (EGFR), roughly 50,000 per cell, affecting a multitude of different signaling pathways [74]. EGFR, a member of the ErbB/HER tyrosine kinase receptor family, can undergo mutations in disease to become constitutively active, the dysregulation of which leads to tumor progression, angiogenesis and metastasis [75]. Binding of EGF to its receptor induces receptor dimerization, phosphorylation of specific tyrosine residues recruit cytoplasmic substrates to activate downstream signaling pathways such as MAPK,

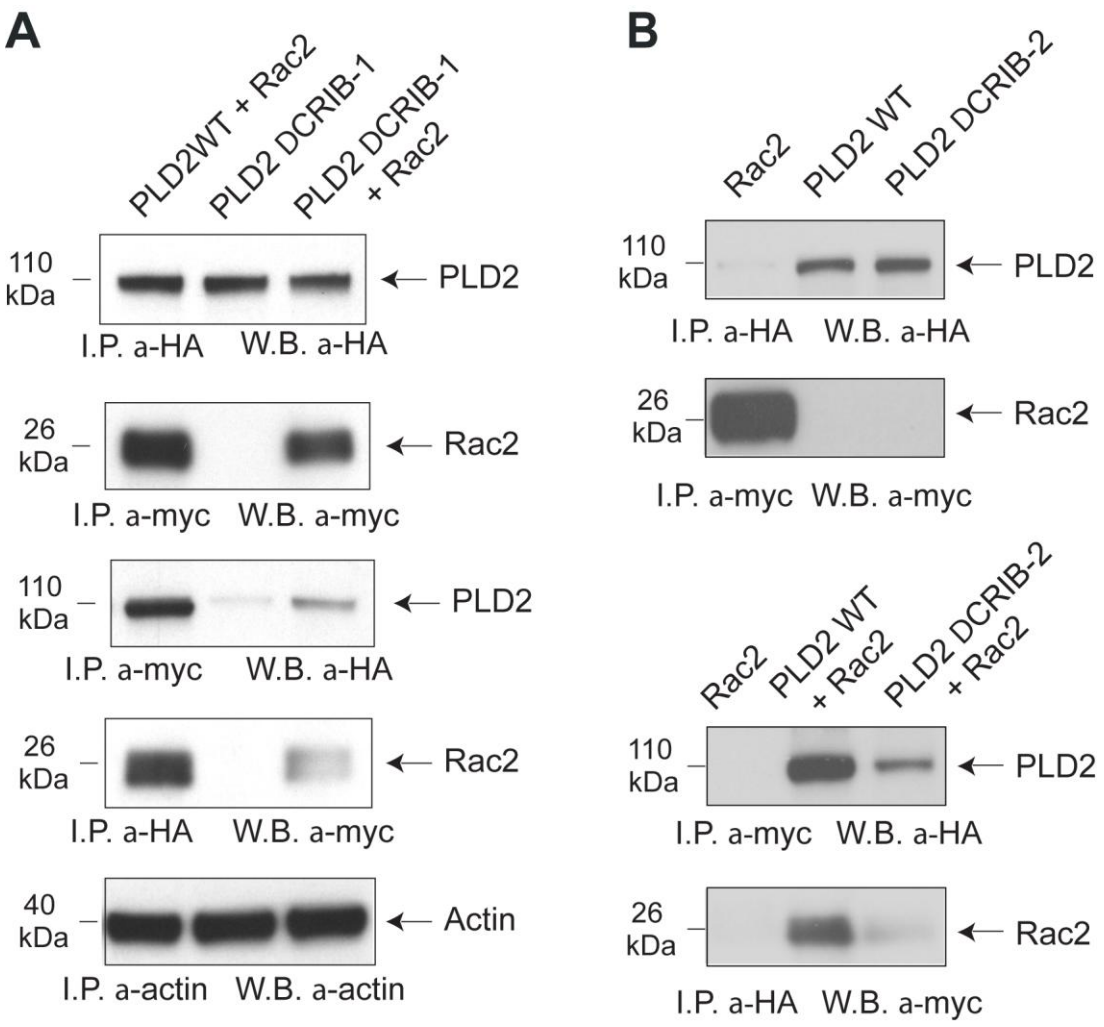


Figure 3: HA-tagged PLD2 and myc-tagged Rac2 were overexpressed in COS-7 cells. By pulling down with immunoprecipitation (IP) and probing for their tags in a Western Blot, we observe the necessity of the CRIB domain for Rac2 binding. In both parts, PLD2 and Rac2 protein are shown. In part A, when DCRIB (deletion of CRIB domain) is overexpressed, Rac2 protein pulldown decreases significantly from the PLD2 + Rac2 co-transfection. Part B examines CRIB2, in which the deletion of that domain again significantly decreases Rac2 protein pulldown, indicating that Rac2 protein expression is dependent on both CRIB domains of PLD2.

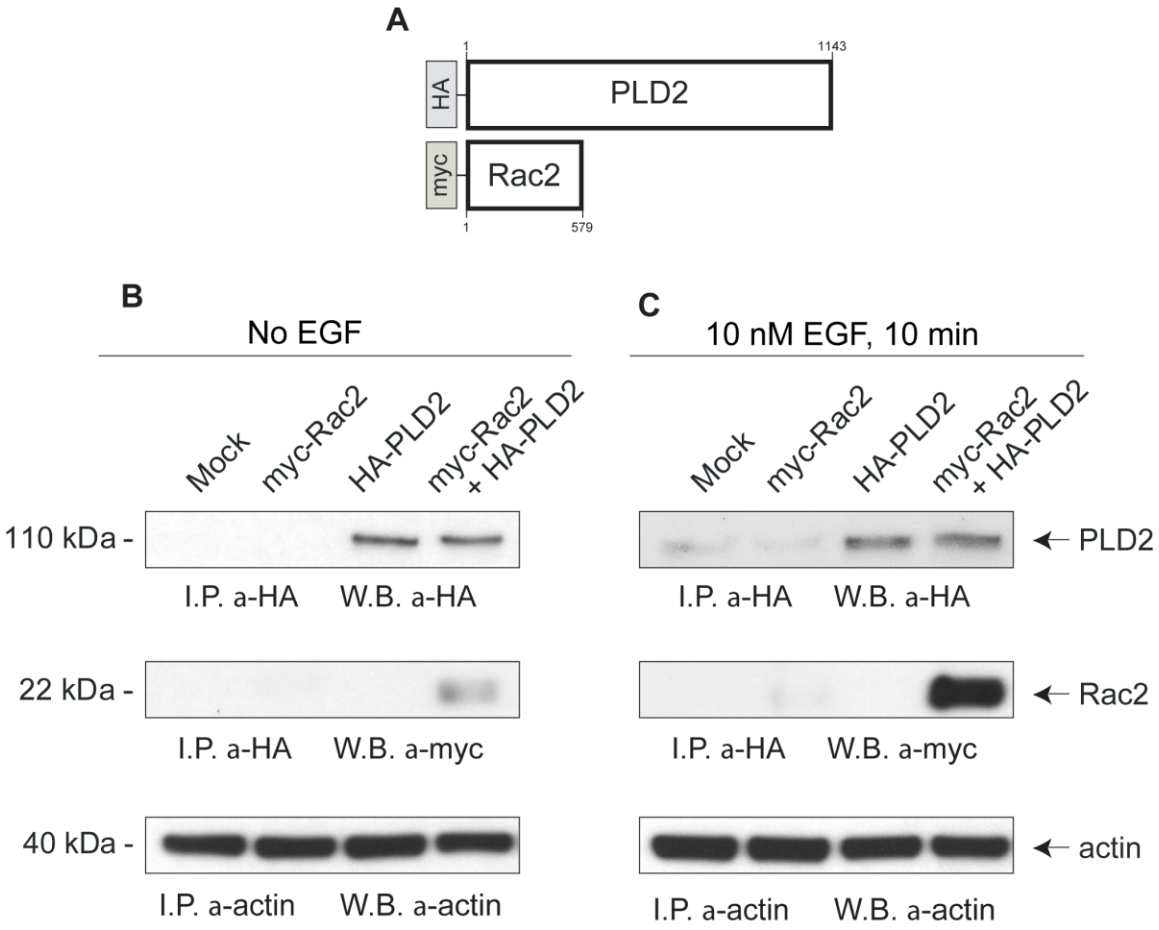


Figure 4: Similar to Figure 3, Rac2 and PLD2 protein interaction is examined in COS-7 cells. Part A illustrates the tags overexpressed by PLD2 and Rac2. Part B, shows that when EGF is not present, Rac2 protein can barely be pulled down. In contrast, EGF-stimulated Rac2, when interacting with PLD2, is pulled down at a much higher level. Therefore, there is an EGF-mediated interaction between PLD2 and Rac2 protein expression.

phosphatidylinositol 3-kinase (PI3K) and signal transducers and activators of transcription (STAT), all critical players in cancer progression [75-78].

MTLn3s are an estrogen receptor (ER) positive cell line. ER are overexpressed in 70% of breast cancers, known as ER+; binding of estrogen to the ER stimulates cell proliferation and an increase in cell division and replication [79,80]. Though we know EGFR overexpression and ER exposure are critical in the pathogenesis of cancer, anti-estrogen drug therapies targeting cell proliferation has shown limited efficacy in deterring tumor progression [4]. MTLn3 as a metastatic cancer is highly prevalent due to its invasive capabilities, which provide another possible mechanism to target other than proliferation. One reason why these cells are so invasive is due in large part to their ability to undergo epithelial mesenchymal transition, in which morphological changes, cytoskeletal restructuring and intracellular E-cadherin redistribution contribute to intravasation [81]. Cell growth and their metabolic processes are heavily influenced by cell shape and cell-cell adhesion/spreading [82]. The maintenance of these cell-cell interactions involve tight cadherin based junctions and cell polarity [81]. Mesenchymal cells dysregulate these interactions and allow invading cells through the ECM through atypical cell surfaces that result in the loss of cell polarity and the synthesis and organization of matrix degrading metalloproteinases (MMPs) [81]. MTLn3 cells in particular have been shown to have high levels of cadherin and proteases (regulators of cell-cell adhesion), low levels of TIMPs (inhibitors of MMPs), loss of intrinsic cell polarity, overexpression of apoptosis supressors and the dramatic polarization of these cells that allow for chemotaxis toward the blood vessels in MTLn3 tumors [83]. The ability for chemotaxis in this microenvironment is due to EGFR signaling from the blood vessels, which involves

small GTPases of the Rho family, enhancing metastatic capablities in addition to the well known effects of EGFR signaling on mitogenesis [83].

Inhibitors

5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) is a pharmalogical inhibitor of PLD that is able to potently and efficiently block *in vivo* PA production by inhibiting the catalytic activity of PLD [84]. In addition, FIPI inhibits PLD regulation of cell spreading, chemotaxis, F-actin cytoskeletal reorganization without delocalization of proteins (such as sequestering PIP_2) or any identifiable effects on other pathways independent of PLD [84].

The flavonoid apigenin (4,5,7-trihydroxyflavone) (Api), is a nonmutagenic bioflavonoid from leafy plants and vegetables that exhibit many different anti-inflammatory, antioxidant and anti-carcinogenic properties, emerging as one of the more promising cancer chemopreventive agents being studied today [85]. Apigenin has been reported to inhibit PKC and MAPK activity [86,87], extracellular signal regulated kinase (ERK) [88], and the decrease of phosphorylated EGFR tyrosine kinase [89]. Dr. Cambronero's lab has proposed that Api may inhibit PLD enzymatic activity and affect PLD2/Rac2 binding due to observed decreases in lipase activity and the increase Rac2 exerts onto it in MDCK cells.

III. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

The objective of the present study is to investigate if PLD2 is mediating cell invasion in mTLn3 cells and the hypothesis is that the mechanism of PLD2-mediated cell invasion either involves protein-protein interaction with other cell signaling molecules, such as Grb2 and Rac2, a dependence on lipase activity, or both.

Specific Aims

In order to test this hypothesis, we outlined a series of experiments within our four Specific Aims:

AIM 1: How does modulation of PLD activity affect invasion? We assayed MTLn3 cells for cell invasion in Matrigel by EGF stimulation over a period of 20 hours. Cell invasion was preceded by transfecting siRNA PLD (for loss of function), as well as overexpressing the Wild-Type (WT) PLD plasmid (for gain of function) and the lipase dead KR mutants before the cells underwent invasion. We also introduced the 5 potential inhibitors to examine their effect on PLD in cell invasion and their effect on PLD enzymatic activity.

AIM 2: To investigate if there is an effect of Grb2 on PLD2-mediated cell invasion and lipase activity. To determine the effect of Grb2 on PLD, we silenced endogenous Grb2WT; we also overexpressed Grb2 WT and the mutants (R86K SH2-deficient and p49/206L SH3-deficient), as well as PLD2 YF (Tyr residue substitutions) mutants (Y169F, Y179F, and Y511F) and followed cell invasion. We examined these plasmid constructs additionally through co-transfections with PLD, enzymatic assays and the effect of the 5 potential inhibitors.

AIM 3: To investigate if there is an effect of Rac2 and CRIB on PLD2-mediated cell invasion and lipase activity. This set of experimental procedures followed the same outline as our Grb2 plasmids. By transfecting our Rac2WT and Rac2 N17 (GDP-bound) plasmids, we were able to investigate their effects on invasion and activity. We also elucidated the role of the PLD-Rac2 interaction in the mTLn3 cancer cell line. Introduction of the PLD2 CRIB domain mutations (in the PH domain), ΔCRIB1 and ΔCRIB2, further defined this interaction.

AIM 4: To investigate the effect of kinase phosphorylation of PLD2 on PLD2-mediated cell invasion and lipase activity. Specific tyrosine residue sites on PLD2 were examined in this study, as kinase activity is known to affect lipase activity of PLD2. Within this aim, we studied the PLD2 YF mutants 296, 415, and 511 that disable kinase interactions and observed their effect on PLD2 in a similar manner as above.

IV. MATERIALS AND METHODS

Materials

The rat mammary adenocarcinoma cell line mTLn3 was a gift from Dr. Jeffrey D. Segall (Albert Einstein College of Medicine). MTLn3 culture medium α-MEM, Lipofectamine 2000, Opti-MEM, Trypan Blue and EGF were all obtained from Invitrogen (Carslbad, CA). The matrigel inserts were purchased from BD (Franklin Lakes, NJ). Hematoxylin stain was obtained from Ricca (Arlington, TX). For silencing, pre-designed siRNA for PLD1 targeting exon 10 and siRNA for PLD2 targeting exon 15 and exon 6-9 were purchased from Ambion (Austin, TX). The siRNA of Grb2 and Rac2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). siQuest was purchased from Mirus (Madison, WI). Negative control siRNA was purchased from Ambion (Austin, TX). ECL reagents used for western blotting were purchased from GE Healthcare (Piscataway, NJ). Maxi-prep plasmid kits were purchased from Qiagen (Valencia, CA). Restriction enzymes were purchased from New England BioLabs (Beverley, MA).

Methods

Cell Culture and Plasmid Transfections.

MTLn3 cells were cultured in α-MEM plus 5% FBS, 50 U/mL Penicillin, 50 µg/mL Streptomycin and 50 µg/mL Gentamycin. Cells were split at 60% confluency. The cells were transfected at 50% confluency per well with plasmids ranging from 1-6 µg of DNA, using 2 µl Lipofectamine 2000 per µg of DNA diluted in Opti-MEM. Sterile glass culture tubes housed each lipid-DNA complex prior to pipetting the solution into 6-well plates of cells containing 2 mL of α-MEM plus 5% FBS only per well. The cells were

then incubated overnight at 37˚ C in a humidified 5% CO_2 atmosphere and the following morning media replaced with fresh complete α-MEM.

Gene Silencing.

For PLD silencing, a negative control and three pre-designed siRNAs were used. PLD1 siRNA targeting exon 10 and PLD2 siRNA targeting exon 15 and exon 6-9. The silencing of Grb2 and Rac2 were also performed (Table 1). The siRNA was transfected by diluting the siRNA in Opti-MEM, followed by the addition of siQuest. Each reaction was incubated for 20 minutes at room temperature before being pipetted dropwise onto the cells. Cells were incubated at 37˚ C in a humidified 5% CO_2 atmosphere and media was replaced after 24 hours. Silencing occurred for 4 days prior to assay.

Cell Invasion.

Cells were serum starved for 2 hours in α-MEM medium supplemented with 12 mM HEPES (MEMH) and 0.1% BSA. This same buffer was used to pre-wet the matrigel inserts (8 micron pores) prior to assaying. The cells were then harvested by disassociating with 3 mM EDTA/PBS, sedimented and counted using Trypan-Blue. Cell viability was uniformly > 95%. $1x10^5$ cells were then loaded into the top of the insert, while the bottom wells contained EGF. The inserts were then incubated for a period of 20 hours and fixed in 10% paraformaldehyde for 15 minutes, followed by staining in hematoxylin for 2 hours. 6 fields within the inserts were then counted by inverted microscopy at 20x magnification and multiplied by a factor of 45 to account for the area of the insert. These values represented an average number of total cells that migrated through the insert. The

siRNA Sequences (5’ to 3’)

siPLD1	GGCAAAUGAAGAGAUUUUU
siPLD2 exon 15	GGACUACAGCAAUCUUAUC
siPLD2 exon 6-9	GGACUCCUUCCUGCUGUAC
siGrb2	CAUGUUUCCCCGCAAUUAU
Negative Control	UGUACUGCUUACGAUUCGG
siRac2	CCACUGUAUUUGACAACUA CACUGGCCAAGGAUAUUGA GAACCAAAGGGAGAGAUGU

Table 1:

The sequences of RNA targeted to silence PLD1, PLD2, Grb2 and Rac2.

error bars are a standard error of the mean, which take into account the differences between cells per field. Inhibition of cell invasion was performed by resuspending the 5 inhibitors in DMSO at 6-10x their IC50 concentrations. The inhibitors were present in the bottom well with the culture media that contains chemoattractant.

PLD Enzyme Activity

Measurement of lipase activity began with the addition of the following reagents: 3.5 mM PC8 phospholipid, 45 mM HEPES at a pH of 7.8 and 1µCi of n-[^{3}H] butanol. Samples were incubated for 20 min at 30˚ C with continuous shaking. The reactions were then stopped with 300 µl of ice-cold chloroform/methanol (1:2). The lipid layer was then isolated and resolved by thin layer chromatography for 50 minutes. The lipids bound to the plate were visualized with iodine vapors. The amount of [^{3}H] PtdBut that co-migrated along with the PtdBut standards was scraped and quantified by scintillation spectrometry. The control reactions contained no PC8, removing background counts. Inhibition of enzyme activity was similar to cell invasion, except the cells were incubated with the inhibitors for 20 minutes in a reciprocal shaking bath prior to assay.

Immunoprecipitation and Western Blotting

After cells were collected, they were then lysed in Special Lysis Buffer (50 mM HEPES, pH 7.2, 100 µM NA_3VO_4, 0.1% Triton X-100 and 5 mg/ml each of aprotinin and leupeptin) and sonicated. BIO-RAD protein assays were utilized to determine protein concentration and normalize for protein per reaction. The samples were then conjugated with anti-myc agarose beads overnight. Immune complex beads were then sedimented,

washed with buffer A (100 mM Tris-HCl, pH 7.4, 400 mM LiCl) then buffer B (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The beads were resuspended in a final volume of 60 µL 4x SDS running buffer for subsequent gel electrophoresis. These gels were then transferred for 1 hour onto PVDF membrane, blocked for 2 hours at room temperature with 2.5% BSA with TBS, 0.2% Tween 20 and probed with primary antibody overnight. Secondary antibodies conjugated to horseradish peroxidase were detected on x-ray films with the use of ECL reagents. The Kodal Gel Logic software used to perform densitometry on Western Blot samples which consists of quantifying the band of the protein of interest versus the band of the actin control.

Maxiprep for DNA Constructs and Restriction Enzyme Digestion

E. coli colonies were grown in LB broth media and processed through the Qiagen maxiprep kit. Once the plasmids were extracted, the concentration and purity were determined by spectrophotometry at OD260/280. The plasmids were then verified by restriction enzyme digestion using 0.4% agarose with ethidium bromide. Gels were electrophoresed at 100V for 1 hour and then visualized by UV transillumination.

V. RESULTS

AIM 1: Is PLD causing invasion in mTLn3 cells?

A. Roadmap of Proteins and Protein Interactions

In Figure 5, we outline the roadmap of this thesis with the proteins involved in this study and their interactions with each other. In Aim 1, we looked exclusively at the effects of PLD1 and PLD2 on cell invasion and enzyme activity, as well as the effectiveness of the 5 potential inhibitors in targeting and affecting the functions of these proteins.

B. Overexpressing Plasmid DNA for Cell Invasion in Matrigels

Initially, we observed the effect of overexpressed WT and mutant PLD plasmids on cell invasion in order to gauge their relative effects. As is the case for every one of the cell invasion assays, we run a mock, unstimulated sample and conversely a mock sample stimulated with EGF. In this particular experiment, we included transfections of our PLD WTs and lipase dead KR mutants with and without chemoattractant. Relatively similar to silencing, the KR mutants deprive PLD of its lipase activity. Without an active lipase, dominant negative cell signaling is interrupted. The results of this initial invasion assay are right in line with what we expected. Our PLD1 and PLD2 WT plasmids, even without EGF stimulation surpass the EGF control (Figure 6a). With chemoattractant, we see a considerable enhancement in invasion with PLD2 WT. PLD1 and PLD2 KR mutant plasmids were utilized and both decreased invasion. In each cell invasion assay presented in this study, a t-test was performed to determine statistically significant increases (*) or

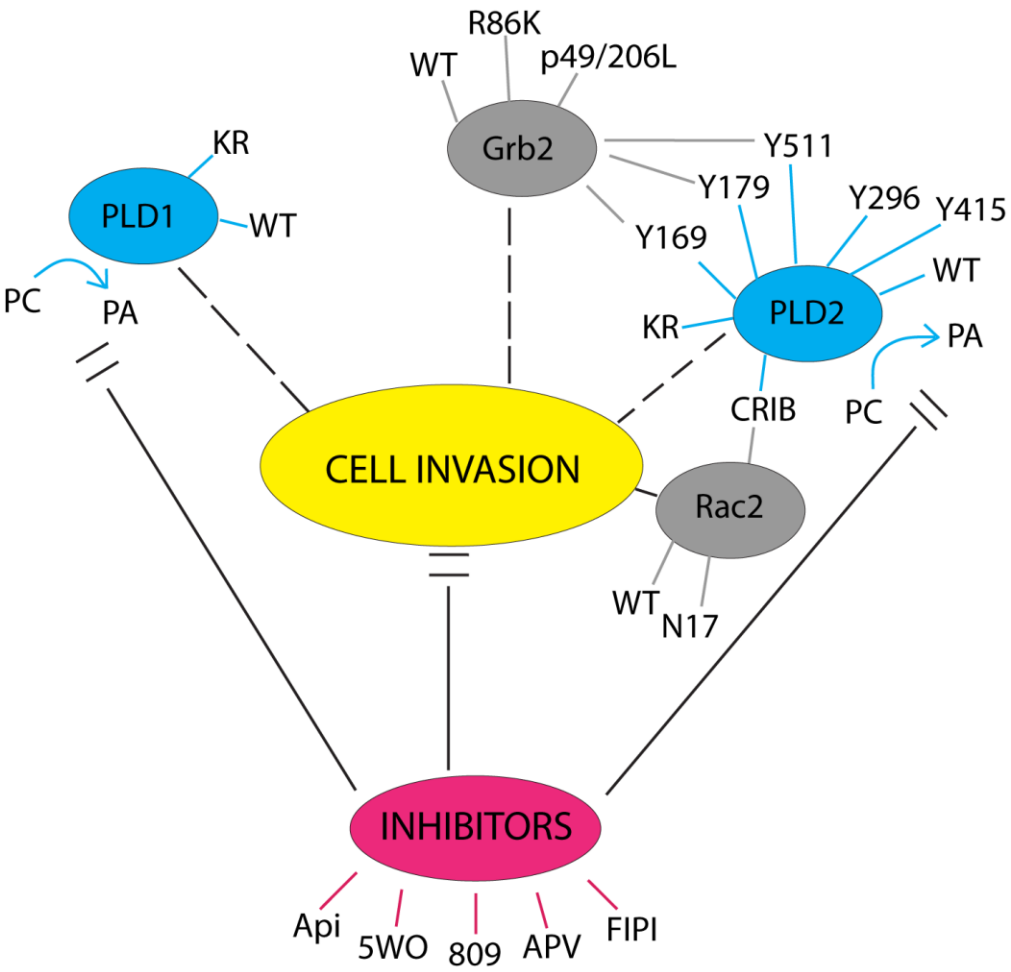
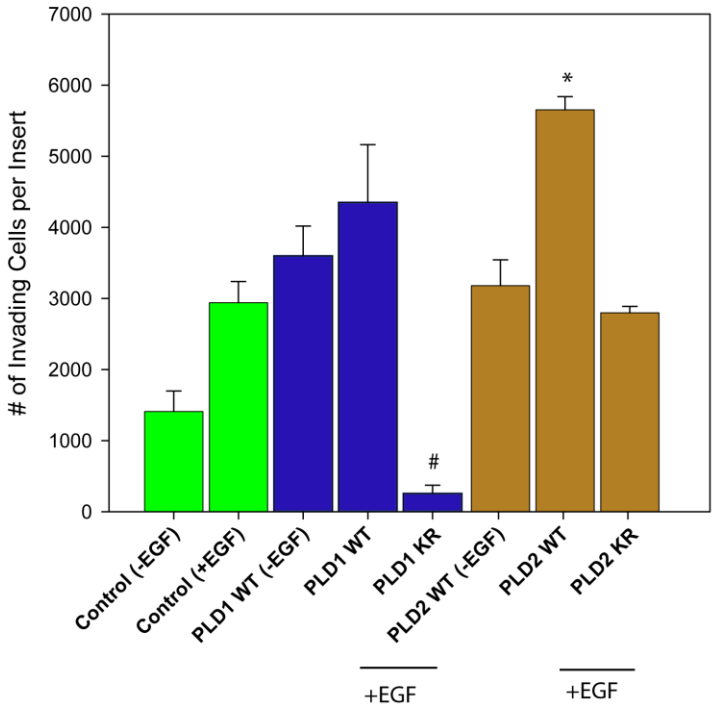


Figure 5:

This roadmap covers the 4 major proteins of study (PLD1, PLD2, Grb2, Rac2) and illustrates the interactions between them. In AIM 1 we examined PLD1 and PLD2 exclusively. The WT or mutant DNA constructed to study that particular protein of interest is linked nearby. In the case of certain plasmids, their effects expand beyond only 1 protein of interest. One such example is the tyrosine residue site Y169 where PLD2 interacts with Grb2. The YF mutants examine these loss of interactions, in this case Y169F.

A.



B.

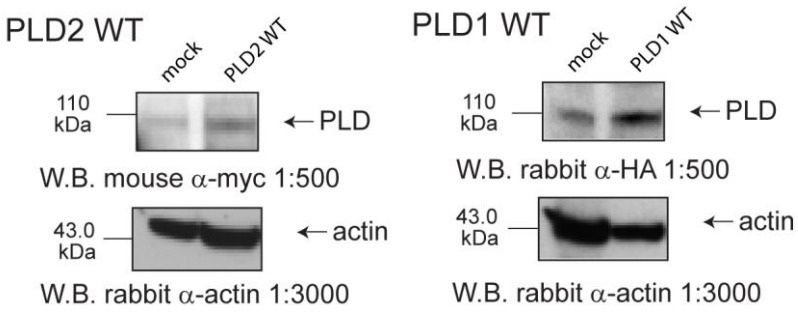


Figure 6:

Part A illustrates the increase in invasion with PLD WT present within the cell, and the subsequent loss of invasion when the lipase dead KR mutants are overexpressed. Part B verifies our overexpression of PLD1 and PLD2 by probing for their tags, HA and myc, respectively. Actin is probed as a loading control.

decreases (#) from the control. In Figure 6a, the p value of PLD2 WT compared to the EGF control is 0.001, any p value less than 0.05 (within 95%), is statistically significant.

C. Silencing Gene Expression.

To verify PLD as essential for cell invasion in MTLn3 cells, we knocked down PLD2 with siRNA before assaying for cell invasion. In order to investigate and verify the loss of gene expression and its effects, we silenced PLD2 in a dose response fashion from 0 nM to 200 nM of siRNA (Figure 7a). We observed subsequent loss of protein expression correlated with the amount of siRNA transfected, indicating a loss of PLD2 expression in our protein samples. Additionally, Figure 7b depicts the densitometry of PLD2 silencing in relation to actin staining as shown in our western blot. Again, we see a consistent loss of protein (up to 90% expression decrease with the highest concentration of siRNA) expressed with increasing amounts of siRNA. The effectiveness of PLD1 silencing was difficult to perform and interpret (data not shown). Now that the efficacy of the PLD2 siRNA has been established, we investigated its effect on cell invasion. If PLD2 is truly essential to invasion, then we expect to see a significant decrease in the ability of MTLn3s to invade through the basement membrane of our matrigel inserts following PLD2 silencing. Accordingly, we observed an 85% decrease in EGF-mediated cell invasion when PLD2 was silenced, confirming the requirement of PLD2s role in cell invasion (Figure 8).

D. Effect of Inhibitors on PLD Mediated Cell Invasion

We must now examine the potency and specificity of the 5 potential inhibitors on

A. **B.**

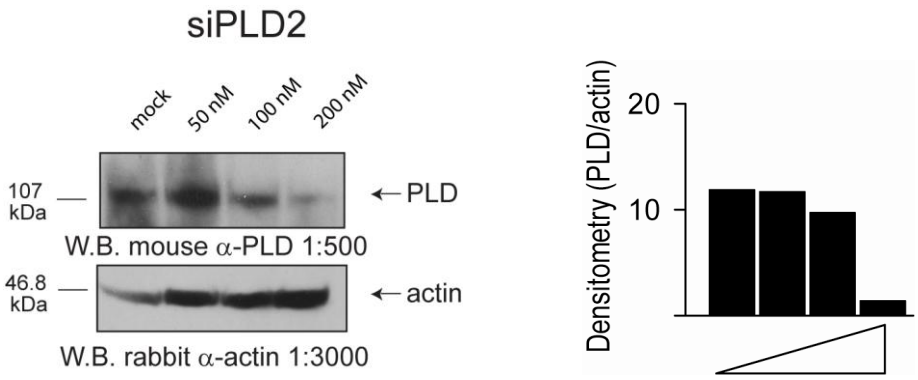


Figure 7:

In Parts A-B we show two different data sets on the effectiveness of PLD2 siRNA. Part A (Protein Expression), Part B (Protein Expression vs. Actin).

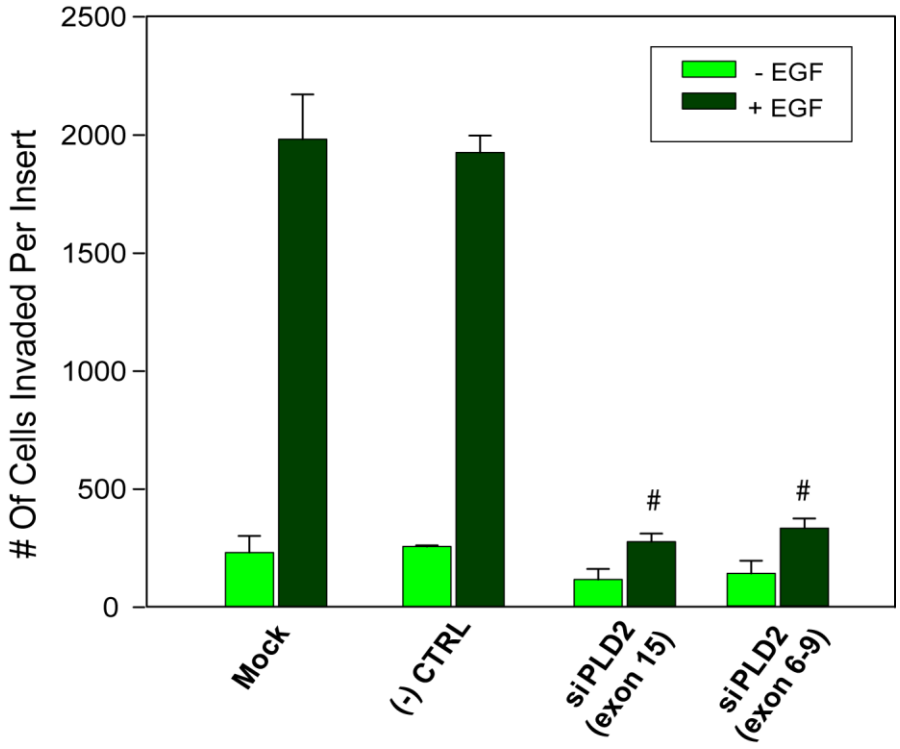


Figure 8:

This graph shows that in the presence of the EGF, PLD2 is crucial for invasion. Compared to the mock, the amount of cells invading drops roughly ~85% when PLD2 is not being expressed. 200 nM siPLD2 exon 15 was used prior in this study, however siPLD2 exon 6-9 was also utilized in this assay to verify that the silencing of PLD2 could also occur by suppressing another part of the gene. This assay also shows that a negative control (a mock transfected condition with siRNA that does not target any particular gene sequence) had a neutral affect on cell invasion. This control eliminates possible background during siRNA delivery, providing a threshold for siRNA effectiveness.

the MTLn3 cell line. First, we performed a dose response cell invasion assay of the inhibitors in MTLn3s with (Figure 9) or without chemoattractant (data not shown). Our goal was to determine IC substrate 50 (IC_{50}) concentrations for each compound. These compounds were inhibitory and largely effective at subnanomolar doses (Table 2). Once we established our IC_{50} for each inhibitor, we investigated their effects on overexpressed PLD1 WT and PLD2 WT (Figure 10). All the compounds excluding FIPI slightly inhibited PLD1-mediated cell invasion with compound APV eliciting the greatest decrease. PLD2-mediated cell invasion on the other hand was more significantly decreased in the presence of all 5 compounds. Api, 5WO, 809, APV and FIPI all inhibited PLD2 mediated invasion approximately 50%. This confirms that the invasive capability of MTLn3 cells overexpressing PLD2 WT can be decreased significantly when exposed to these inhibitors *in vitro.* In this case, PLD2 was targeted and knocked down much more effectively than PLD1.

E. Effect of Inhibitors on PLD Enzymatic Activity

The 5 inhibitors, to be truly effective, must possess the ability to inactivate PLD catalysis. If the effects of cell invasion are consistent with the effects on activity, then it is likely only the PLD pathway is being affected. An enzymatic assay of PLD was performed with the same conditions used for cell invasion. (Figure 11). Insignificant inhibition of PLD1 activity occurred in the presence of the 5 inhibitors. However, in the case of PLD2, lipase activity was decreased 30% in the presence of Api, 809, or APV, 40% in the presence of 5WO and 50% in the presence of FIPI.

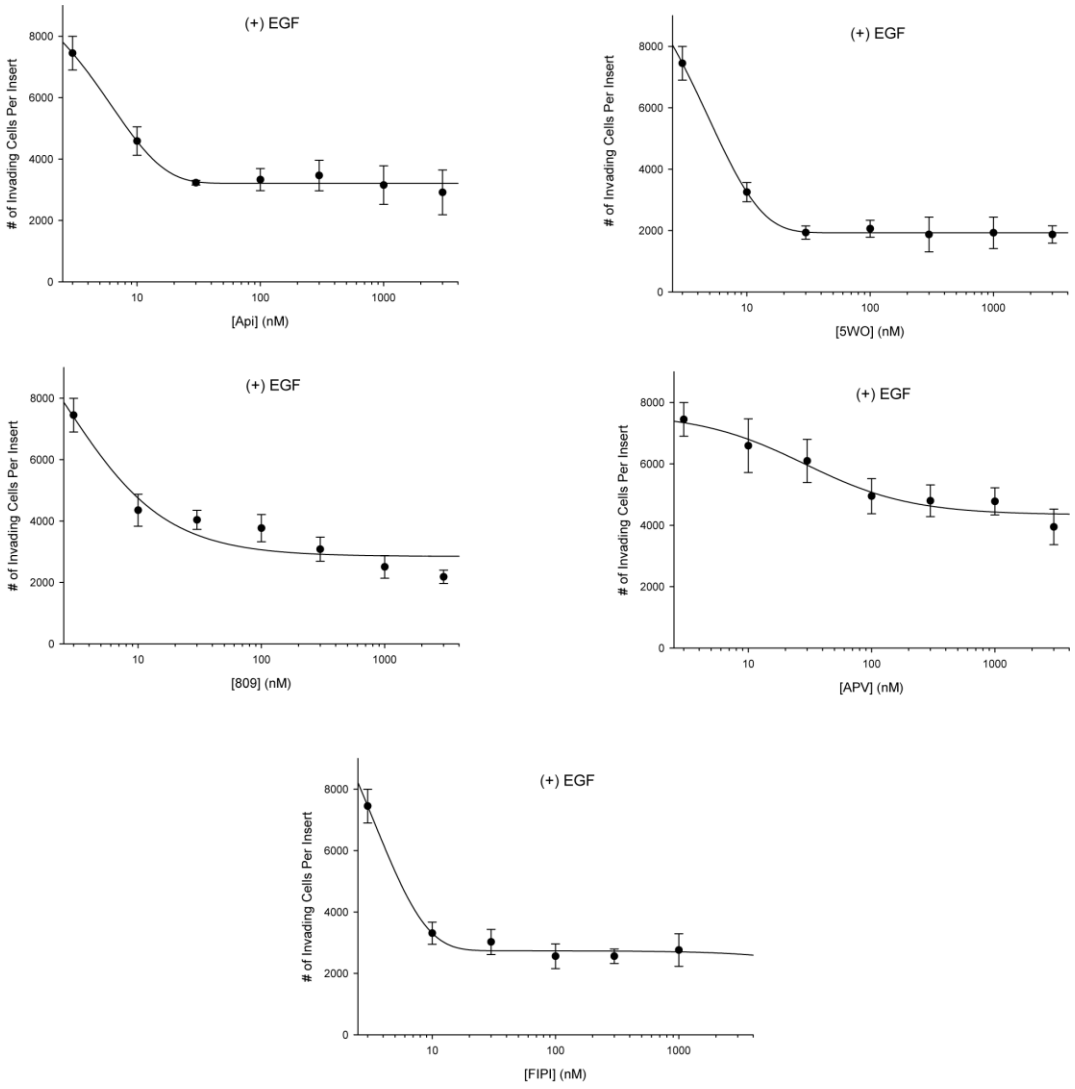


Figure 9:

The effect of the 5 potential inhibitors on cell invasion in the presence of EGF is shown here. These plots were used to determine IC_{50} concentrations for optimal utilization in cell invasion inhibitor assays. Each IC_{50} concentration was determined from the graphs at the midpoint of optimal inhibition, the working concentration used in each inhibitor assay is 6-10x that amount. The cells can still migrate with higher doses of inhibitor present, indicating the inhibitors are effective only to a certain degree.

IC_{50} Concentrations of the 5 Potential Inhibitors

Cell Invasion Assay Values:

Compound	**IC_{50}** (nM)	**Working Concentration** (nM)
Apigenin	5	30
5WO	4.5	30
809	6.5	100
APV	30	300
FIPI	4.5	30

Table 2:

The MTLn3 cell line underwent a dose response cell invasion assay with increasing amounts of inhibitors. $ICsubstrate_{50}$ (half maximal inhibitory concentration) is the functional strength of the inhibitor, measuring the effectiveness of the compounds to inhibit biological functions.

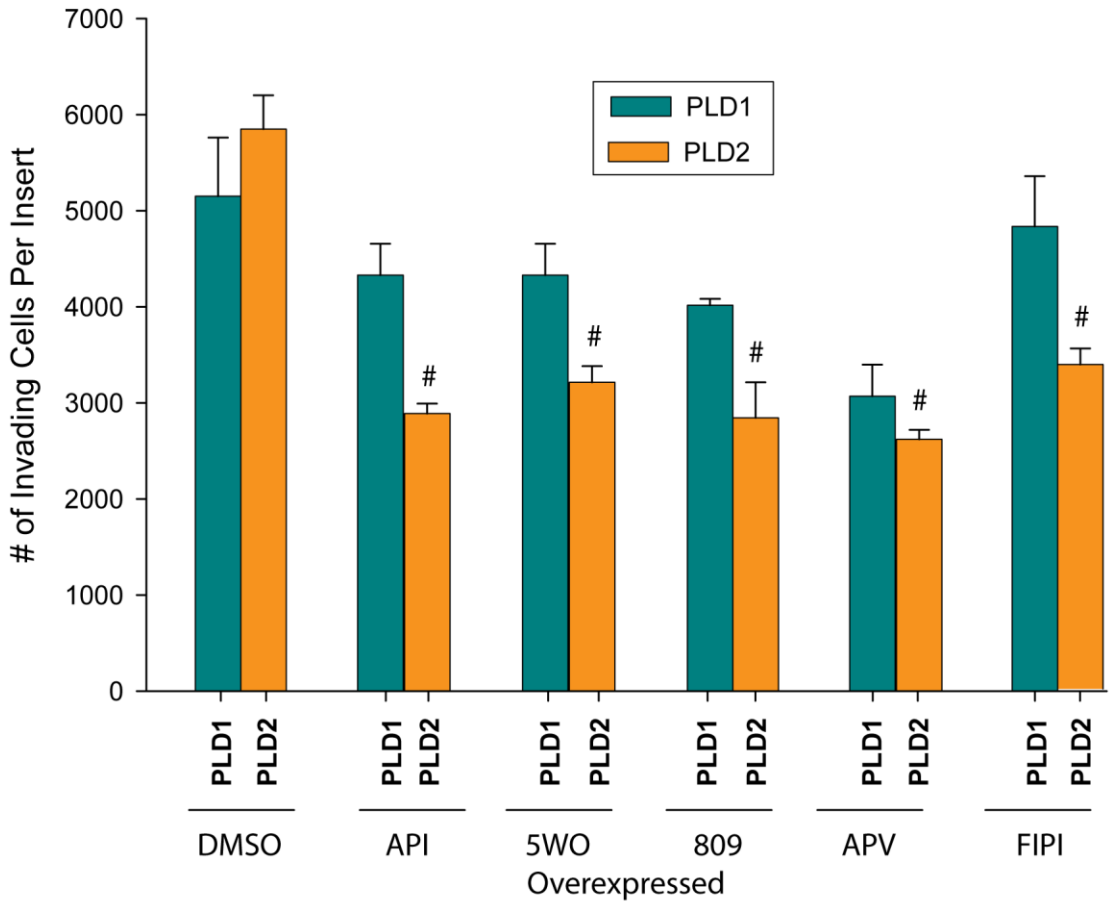


Figure 10:

The effect of the inhibitors on PLD mediated cell invasion. PLD1 is relatively unaffected while PLD2 exhibits ~50% inhibition. There is also a control DMSO group to account for its effect (all inhibitors are resuspended in DMSO), which is run in every assay involving the inhibitors. When PLD is not overexpressed, untransfected cells are inhibited similar to the IC50 graphs, but significantly less that that of overexpressed PLD2 WT (data not shown). In every inhibitor assay the 5 compounds are used at working concentration.

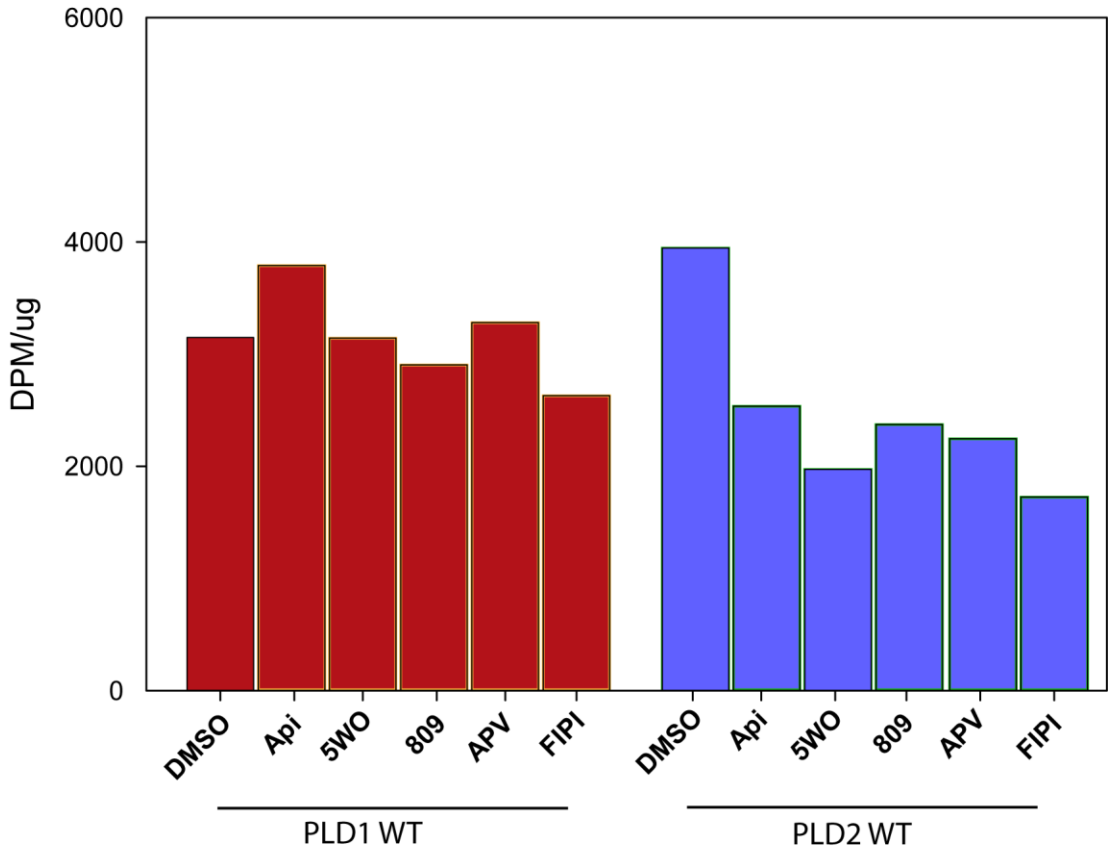


Figure 11:

Each enzyme assay presented is in DPM/µg, disintegrations per minute corrected for total protein. This PLD enzymatic activity assay shows a direct effect on the catalytic activity of PLD2 by the 5 inhibitors. Similar to the effects seen in cell invasion, PLD2 experiences ~50% inhibition. PLD1 is unaffected. Only with FIPI do we begin to see an affect, and in the case of apigenin, we actually see a small spike in activity. We repeated additional independent experiments with the inhibitors on PLD WT and observed a similar trend on lipase activity.

AIM 2: Investigate if there is an effect of Grb2 on PLD2-mediated cell invasion and lipase activity.

A. Roadmap of Proteins and Protein Interactions

In the second aim, we investigated the protein-protein interaction between Grb2 and PLD2 on cell invasion and enzymatic activity. We also investigated the effect of inhibitors on the PLD2-Grb2 association (Figure 12).

B. Overexpressing Plasmid DNA for Cell Invasion in Matrigels

Our next step was to observe the overexpressed plasmids of Grb2 and their effects on cell invasion. In addition to our Grb2 WT, we used the SH2-domain deficient R86K mutant, as well as the SH3 domain-deficient double mutant p49/206L. Assaying these plasmids in cell invasion, we were able to observe the effect Grb2 has on PLD2-mediated cell invasion (Figure 13).

C. Overexpressing PLD2-Grb2 YF mutants in Cell Invasion

PLD2 can bind to Grb2 at the tyrosine sites Y^{169}, Y^{179} and Y^{511} [21,66]. The YF mutants at these sites render PLD2 incapable of binding Grb2. Figure 14a is the 3 YF mutants overexpressed in a Western Blot and Figure 14b the result of cell invasion. As expected, the loss of PLD2's ability to bind to Grb2 results in significant decreases in cell invasion.

D. Silencing Gene Expression.

To investigate the importance of the PLD2-Grb2 interaction in cell invasion, we

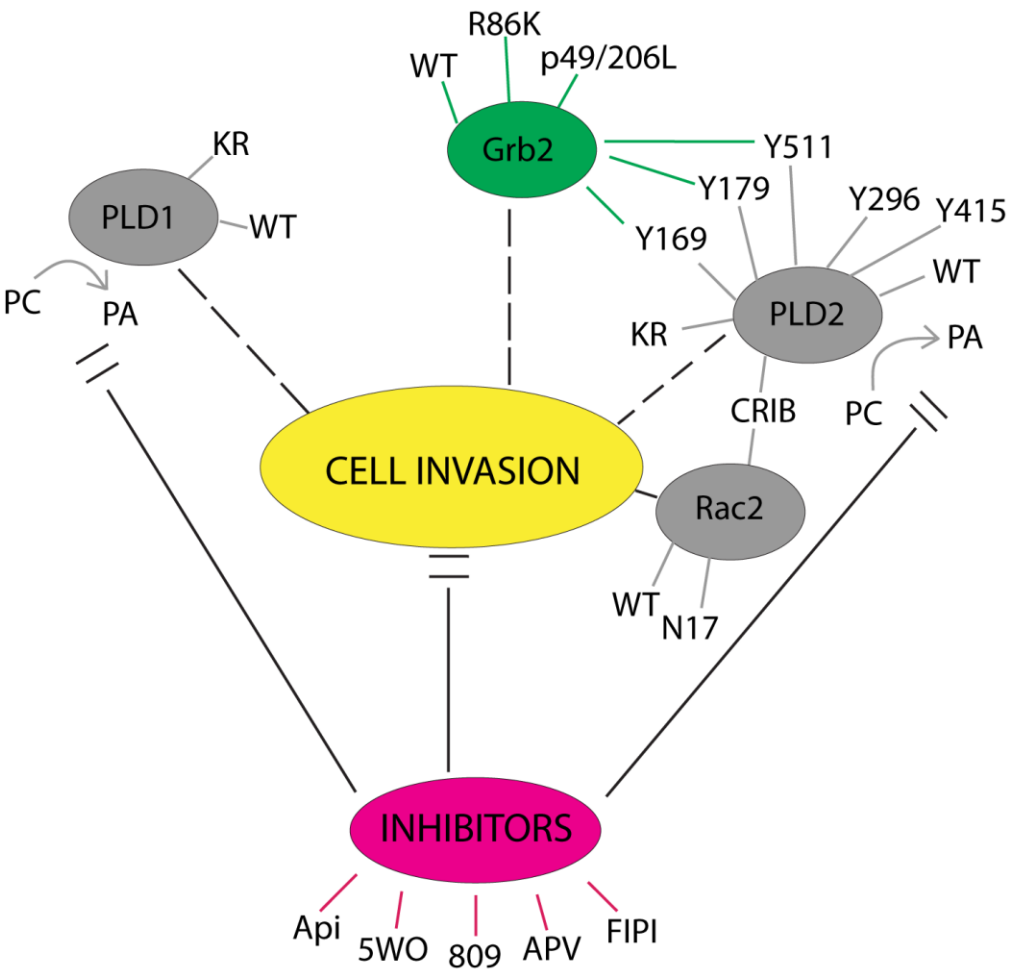


Figure 12:

The next area of study within the roadmap is the effect of Grb2 on PLD2 mediated cell invasion and lipase activity. The specific tyrosine residue sites for protein-protein interaction between these two proteins are Y169, Y179 and Y511 on PLD2 (indicated by the green lines). Grb2WT, Grb2 mutants and PLD2 YF mutants will be used to determine the role of Grb2 in cell invasion (dotted lines). The inhibitors effect on Grb2 and its interactions will also be investigated.

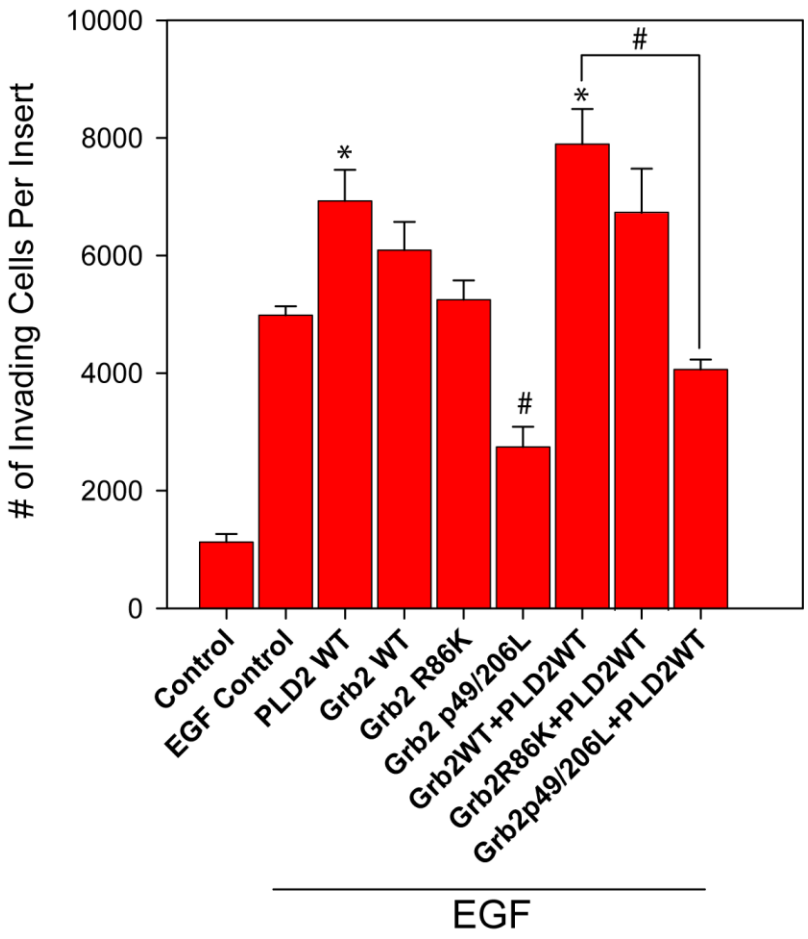


Figure 13:

Grb2 WT increases invasion above the EGF control, though its effects are not as strong as PLD2WT. The Grb2 R86K mutant shows no effect. The double Grb2 mutant p49/206L has a negative effect on cell invasion. The Grb2WT + PLD2 WT co-transfection enhances cell invasion, while our Grb2 R86K + PLD2WT co-transfection shows no increase from that of the PLD2 WT control sample. The addition of PLD2WT to Grb2 p49/206L does not change the negative effect of this Grb2 mutant on cell invasion.

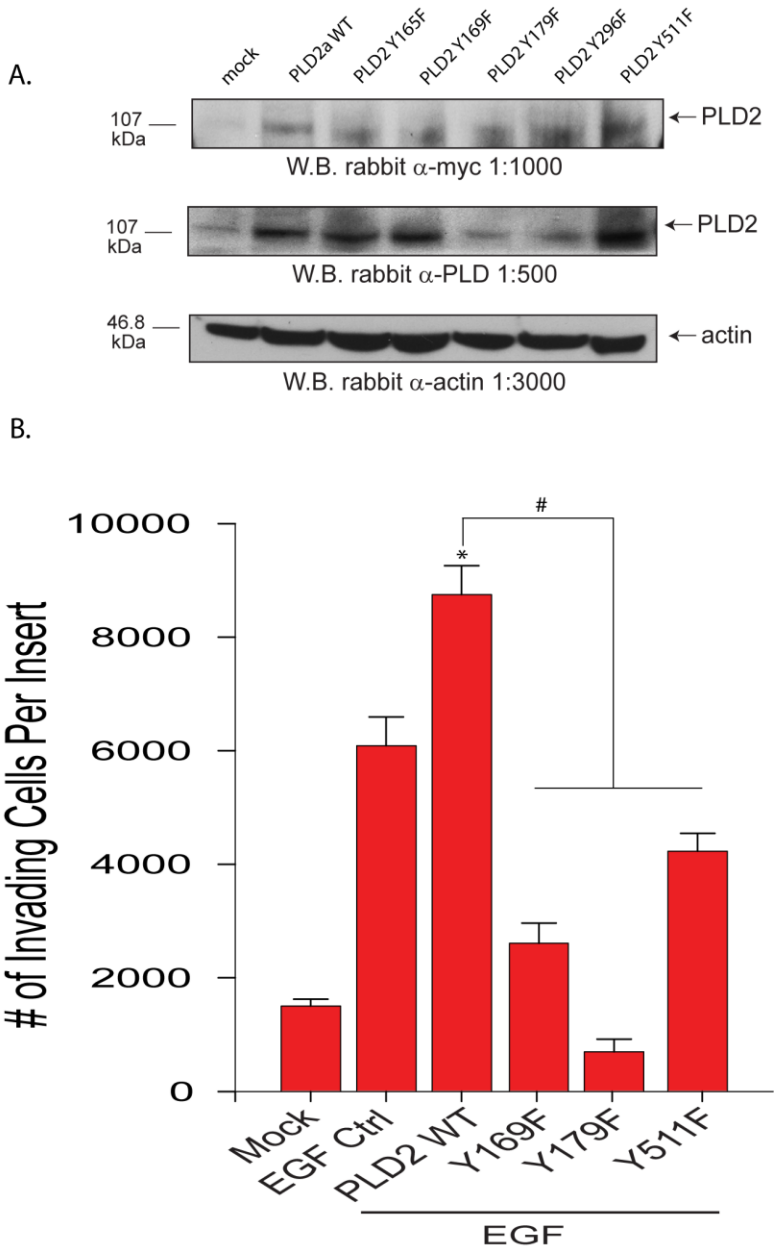


Figure 14:

Part A is a Western Blot of the overexpressed mutants, which were also probed for endogenous PLD to verify overexpression. Part B illustrates the decrease in cell invasion by preventing PLD2 binding to Grb2 when the YF mutants are overexpressed . (Y165F data not shown, Y296F data presented later)

performed a dose response of siGrb2 RNA in the same manner as for PLD (Figure 15a). Additionally, we also quantified the silencing using densitometry (Figure 15b). The siRNA proved to be effective, knocking down Grb2 protein expression levels by over 90% in relation to increasing levels of siRNA. By successfully knocking down Grb2, we can determine the importance of the role it plays on PLD2 in mediating cell invasion. We silenced Grb2 alone or in combination with PLD2 silencing and then assayed for cell invasion (Figure 16). The results are clear that silencing Grb2 only negatively affects the ability of MTLn3 cells to invade. siPLD2 alone has a greater negative effect than siGrb2 alone. Because of the importance of the PLD2-Grb2 interaction, simultaneously knocking down the gene expression of both PLD2 and Grb2 lowers the cell invasion to less than basal levels.

E. Silencing and Rescuing Protein Expression

To examine this interaction further, we immunoprecipitated (IP) Grb2, PLD2 and co-transfected PLD2-Grb2 samples by pulling down PLD2 with α-myc agarose beads and performing a Western Blot to probe for interaction with Grb2 and determine protein expression. By silencing PLD2 first and then overexpressing the same conditions to observe the effect siPLD2 had on Grb2, protein expression is similar in both the Grb2 WT only and PLD2 WT + Grb2 WT co-transfection (Figure 17). PLD2 does not control the gene expression of Grb2; the effects of this interaction are likely further downstream.

F. Effect of Inhibitors on Grb2's Role in PLD Mediated Cell Invasion

After observing the effects of overexpressed Grb2WT in cell invasion, we

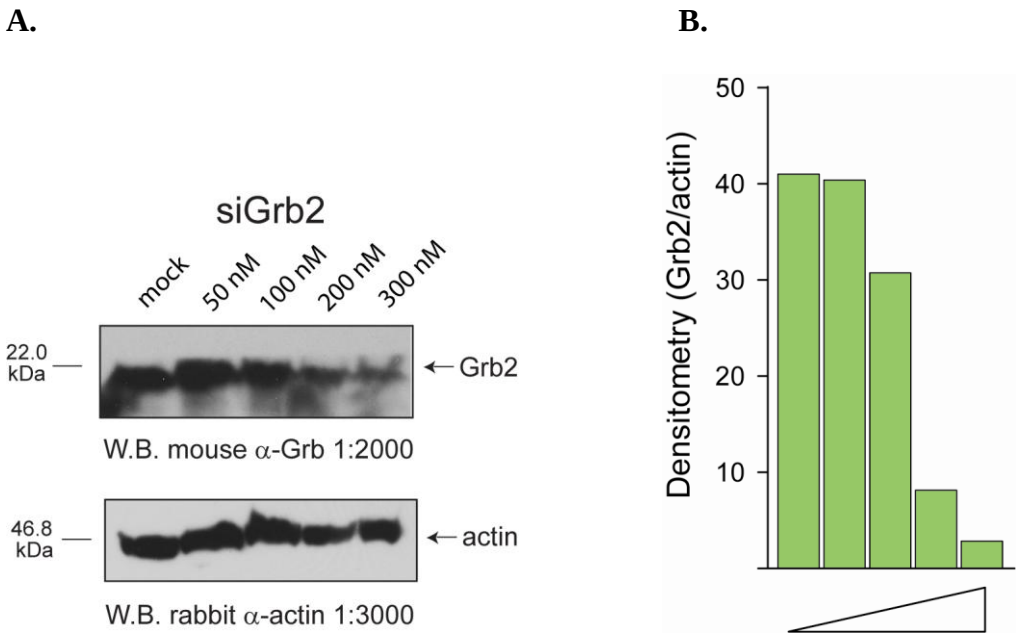


Figure 15:

Part A shows the decrease in protein expression of siGrb2 in a Western Blot. Part B is the densitometry measured from the same blot. A substantial decrease in the Grb2 protein being expressed is observed.

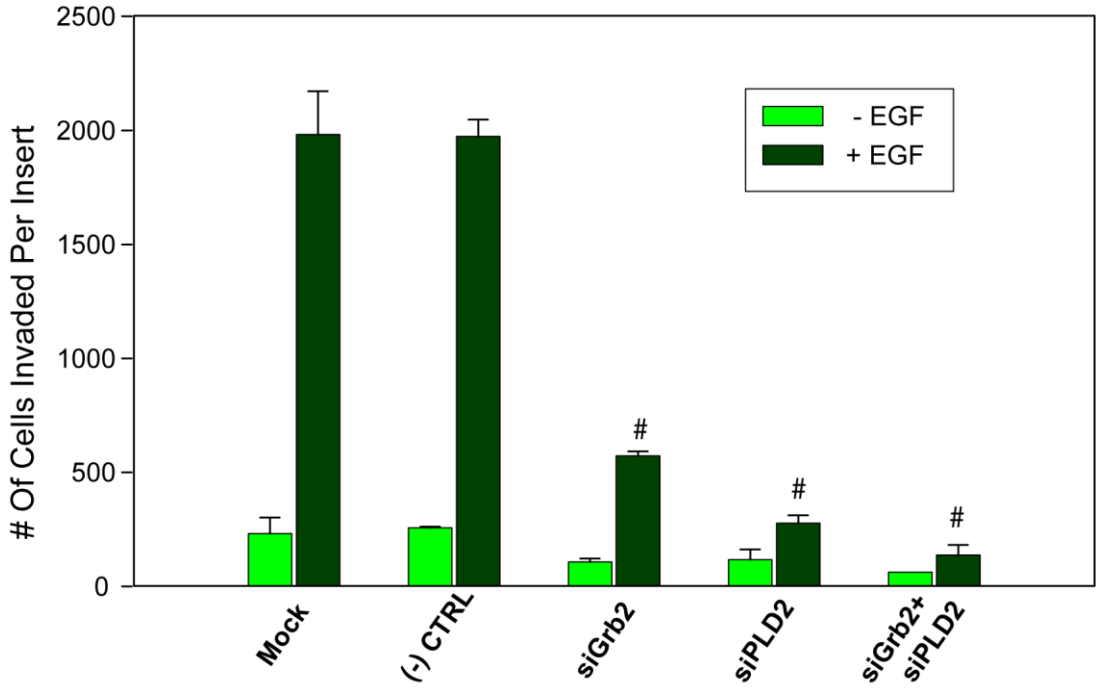


Figure 16:

This silencing invasion assay illustrates the negative effects on invasion when silencing Grb2 or PLD2 alone and most effectively the PLD2 + Grb2 co-silencing. When neither of these genes is expressed, cell invasion is almost completely abrogated.

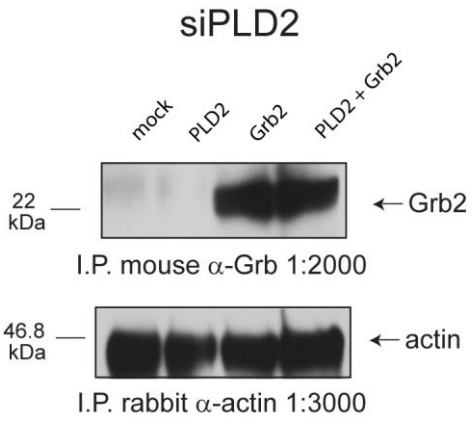


Figure 17:

Grb2 WT and PLD2 WT were overexpressed and immunoprecipitated. PLD2 was silenced prior to transfection for protein overexpression. The similarity in protein expression between Grb2 WT only and PLD2 WT + Grb2 WT indicate PLD2 has no effect on Grb2 protein expression.

investigated the extent to which the inhibitors would knock it down. We also did this with the Grb2WT + PLD2 WT co-transfection, a strong performer in cell invasion (Figure 18). We see roughly a 60% inhibition with Api, 5WO, 809 and APV on our 3 transfection sets. FIPI inhibits the PLD2 + Grb2 combination by ~70%, the first sign of FIPI being the strongest inhibitor. Some of this effect might be attributed to less PLD2 WT overexpressed in the co-transfected sample.

AIM 3: To investigate if there is an effect of Rac2 and CRIB on PLD2 mediated cell invasion and lipase activity.

A. Roadmap of Proteins and Protein Interactions

In the third aim, we investigated the protein-protein interaction between Rac2 and PLD2 and its effect on cell invasion and enzymatic activity. We also examined the effect of the inhibitors on the Rac2-PLD2 association, as well as the effect the CRIB domain has on this protein-protein interaction (Figure 19).

B. Overexpressing Plasmid DNA for Cell Invasion in Matrigels

Figure 20a depicts overexpressed Rac2WT in a Western Blot. To determine the role of Rac2 in cell invasion, we utilized both the Rac2 WT and Rac2 N17 mutant to investigate the effect of Rac2 GTPase activity on cell invasion (Figure 20b). This assay clearly showed that GDP-bound Rac2 N17 has a positive effect on cell invasion above both the mock sample and Rac2WT. We discovered that GTP-bound Rac2WT has a neutral to slightly negative effect on cell invasion. Rac2 N17 is a better performer in cell invasion as GDP-bound Rac2 does not compete with the binding of PLD2 to PIP2 on the cell membrane, as they both bind at the same site on PLD2.

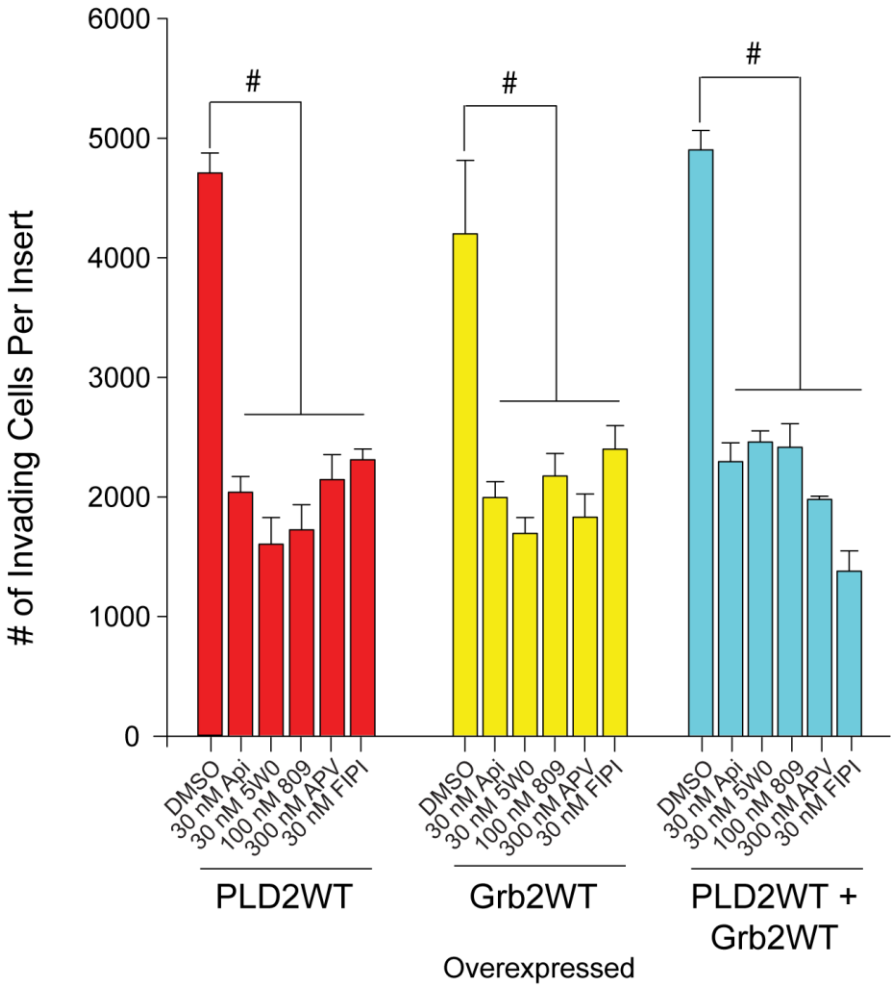


Figure 18:

For cell invasion assays using the inhibitors to determine the protein-protein interaction between PLD2 and Grb2, we use PLD2WT as a positive control. We see ~60% inhibition with Api, 5WO, 809 and APV. Fipi inhibits the PLD2 + Grb2 co-transfection by ~70%. This is the first sign of FIPI as the strongest inhibitor, likely due to its ability to inhibit PA produced by the interaction of PLD2 and Grb2 in the cell.

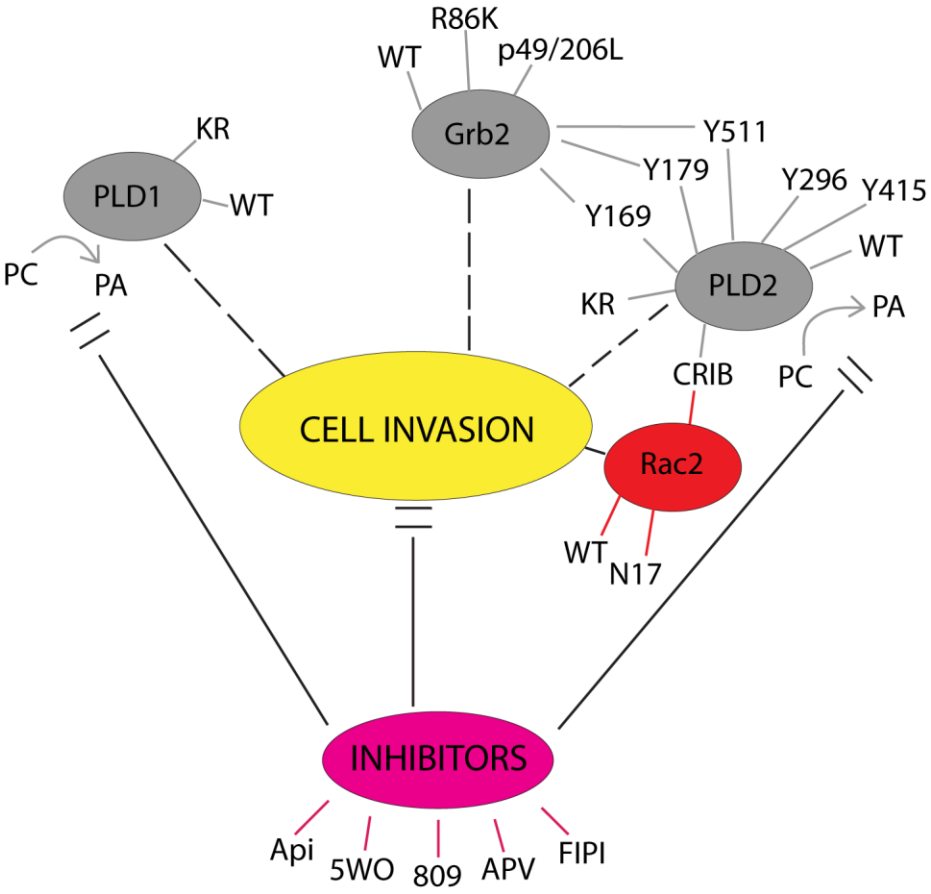


Figure 19:

To determine the role of Rac2-mediated cell invasion and lipase activity we investigated the mutant Rac2 N17, the effect of Rac2 WT and its interaction with PLD2, as well as the effect of the Rac2 binding target located on PLD2 (CRIB).

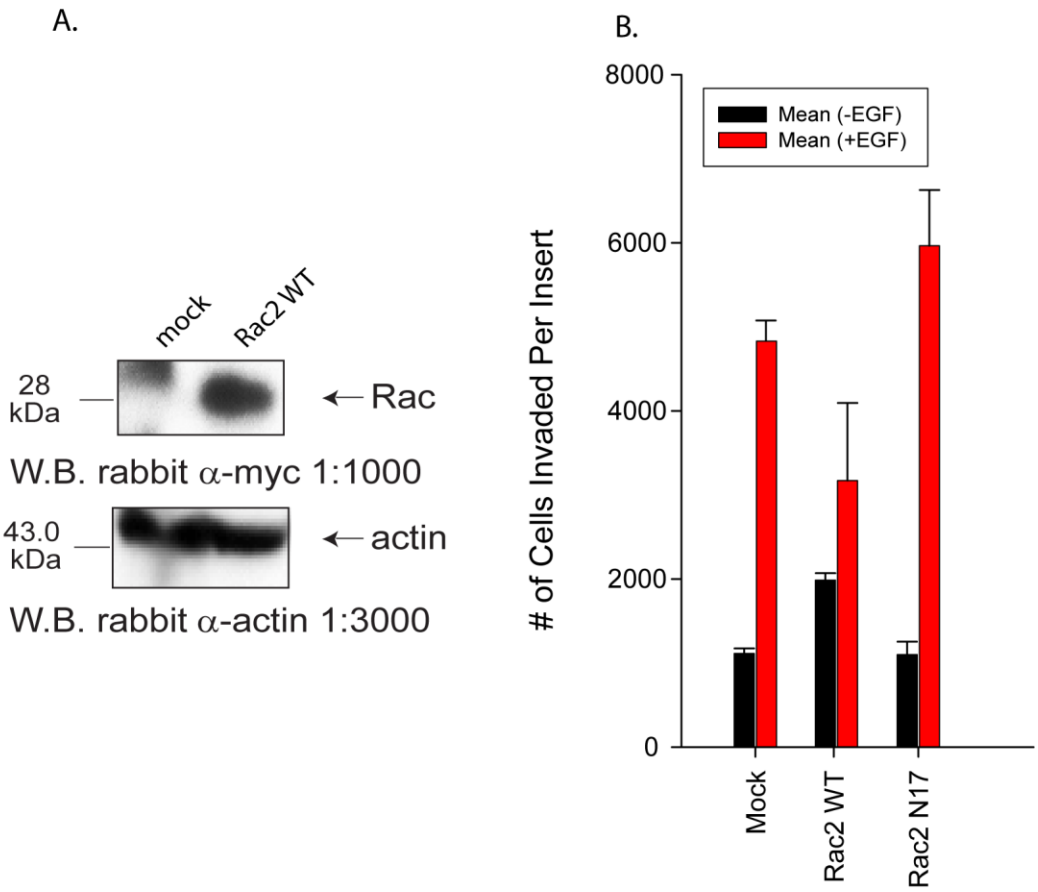


Figure 20:

Part A is a Western Blot of overexpressed Rac2 WT. Part B is the cell invasion data depicting the slightly negative effect of Rac2WT and the positive effect of the mutant Rac2 N17 over Rac2 WT.

The next step was to elucidate Rac2's interaction with PLD2 in a sequentially transfected cell invasion assay (Figure 21). Co-transfecting PLD2WT + Rac2WT simultaneously resulted in a slight decrease in cell invasion below the positive PLD2 WT control. Subsequent transfections of PLD2WT + Rac2WT were performed in sequence and not simultaneously and for differing periods of time. Regardless of the time frame used during sequential transfection, PLD2WT transfection before Rac2WT transfection resulted in an increase in cell invasion.

C. Rac2-Mediated Cell Invasion and PLD Enzyme Activity

To further determine the effect of Rac2 on PLD2-mediated cell invasion, we performed a cell invasion assay (Figure 22a) and a PLD enzymatic assay (Figure 22b) of simultaneously co-transfected samples. Both assays were transfected with the same conditions, increasing amounts of Rac2 WT in the absence or presence of co-transfected PLD2 WT. In cell invasion, the negative effect of Rac2 is descending in effect with concomitant increasing amounts of Rac. When Rac2 WT is co-transfected with PLD2 WT, this interaction results in a more pronounced negative effect on cell invasion when compared to Rac2 only samples, indicating MTLn3 cells become less invasive when more Rac2 WT is present. The effect of Rac2 on PLD2 lipase activity further confirmed this negative interaction. When Rac2 WT was co-transfected with PLD2 WT, the catalytic activity of PLD2 decreased substantially. This pattern confirms that the negative effect of Rac2 on cell invasion is due to the inhibition of both endogenous and overexpressed PLD2 enzymatic activity. As Rac2 levels increase within the cell, PLD2 catalysis becomes less active which contributes to the decrease in cell invasion.

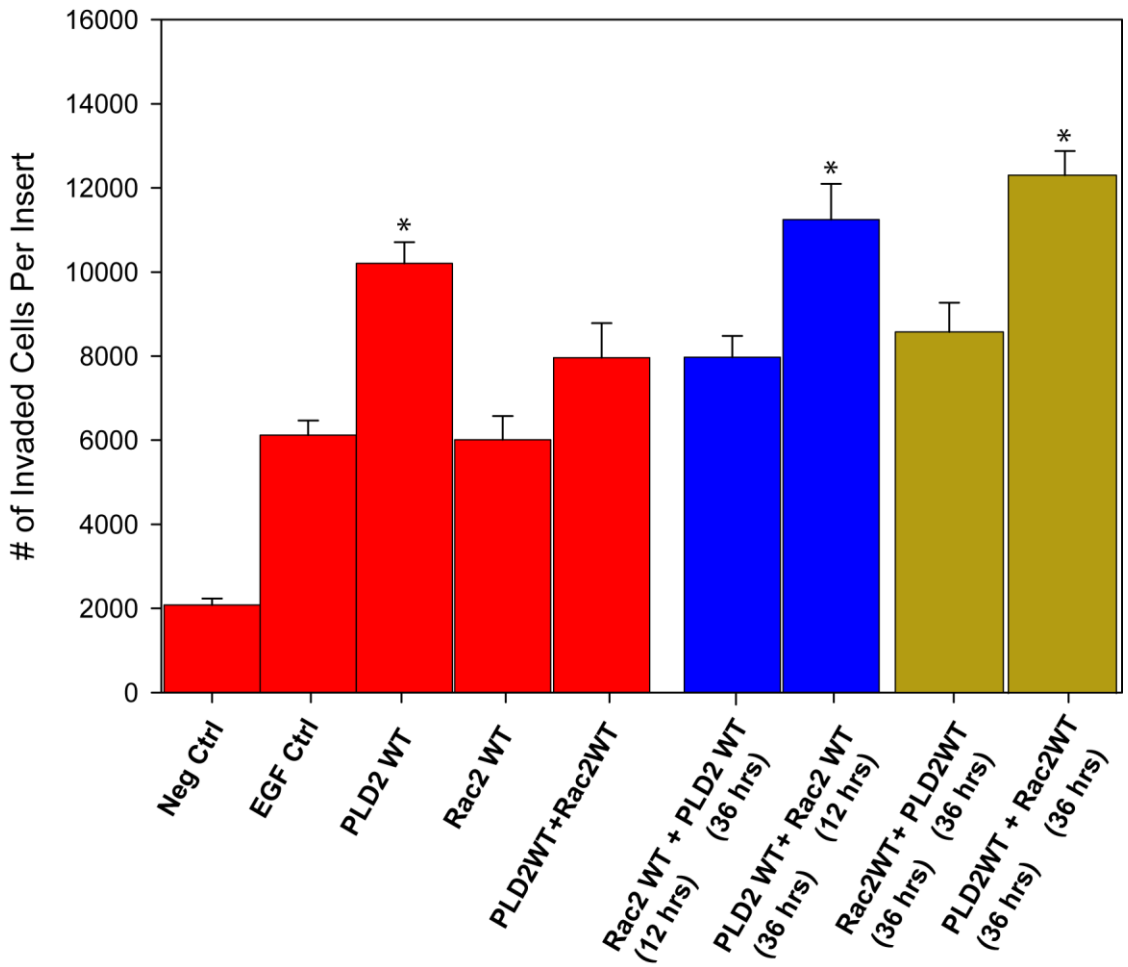


Figure 21:

The simultaneous transfection of PLD2 WT + Rac2 WT is the last bar in red in the middle of the graph and results in a slight decrease in cell invasion when compared to PLD2 WT alone. Additionally, this negative effect on cell invasion is also seen in the sequentially transfected samples when Rac2 WT is transfected before PLD2 WT. When PLD2 WT is transfected before Rac2 WT, the inverse effect is observed and invasion significantly increases above the EGF control.

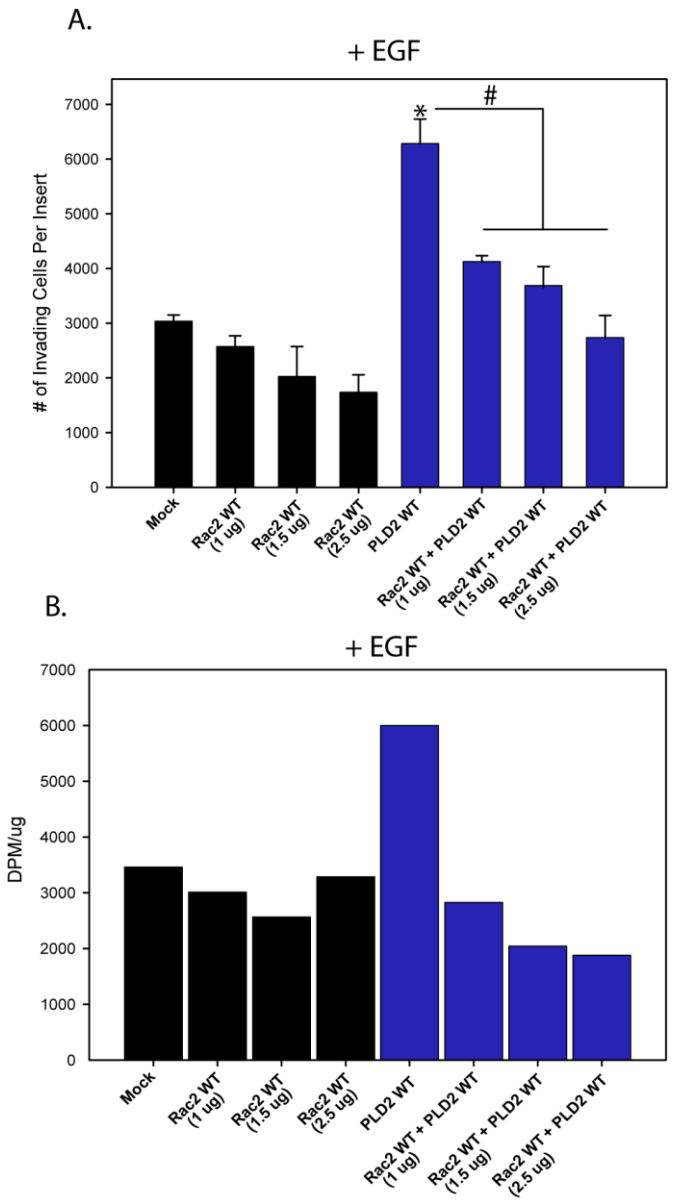


Figure 22:

Part A depicts the decrease in cell invasion when Rac2 WT is co-transfected with PLD2 WT. This effect from Rac2 on PLD2 is confirmed in our enzyme activity assay (Part B). The black bars on the left show the negative to neutral effect of Rac2 on endogenous PLD activity. Recombinant PLD2 enzyme activity is decreased to endogenous levels when interacting with Rac2 WT, overcoming the highly catalytic overexpressed PLD2 WT.

D. Silencing Gene Expression.

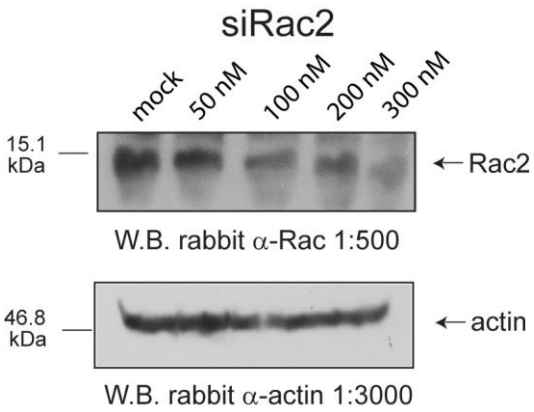
We verified siRac2 in a dose response Western Blot (Figure 23a), as well as quantifying the protein expression using densitometry (23b). As before, our protein expression decreased in proportion to increasing amount of siRNA.

E. Silencing and Rescuing Invasion

We performed a silence and rescue cell invasion assay involving siRac2 and siPLD2 in an effort to define a role for Rac2-mediated cell invasion (Figure 24). In the presence of chemoattractant, the result of silencing PLD2 was a 70% decrease in cell invasion, reinforcing the significance of PLD2 in cell invasion. siRac2 decreased cell invasion by 30%, indicating a role for Rac2 independent of PLD2 in mediating cell invasion. With no EGF stimulation to the cells, the effects of silencing Rac2 plus PLD2 overexpression were more negatively pronounced than silencing of Rac2 alone. Overexpressed PLD2WT was able to rescue EGF-stimulated cell invasion after silencing Rac2. EGF-dependent cell invasion increased 2-fold compared to siRac2 alone. This increase in cell invasion indicates a possible PLD2/Rac2 interaction mediated through EGF stimulation.

To determine if the gene expression of PLD2 affects protein expression of Rac2, we examined the effect of silencing PLD2 and subsequently overexpressing PLD2, Rac2 and PLD2 + Rac2 by performing an immunoprecipitation and probing for Rac2 (Figure 25). PLD2 was only able to partially rescue Rac2 protein expression.

A.



B.

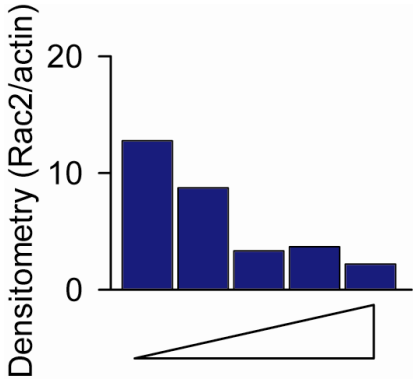


Figure 23:

Part A illustrates the decrease in Rac2 protein expression following silencing in a Western Blot. Part B is the densitometry measured from the same blot. Both data sets show a clear decrease in Rac2 protein expression.

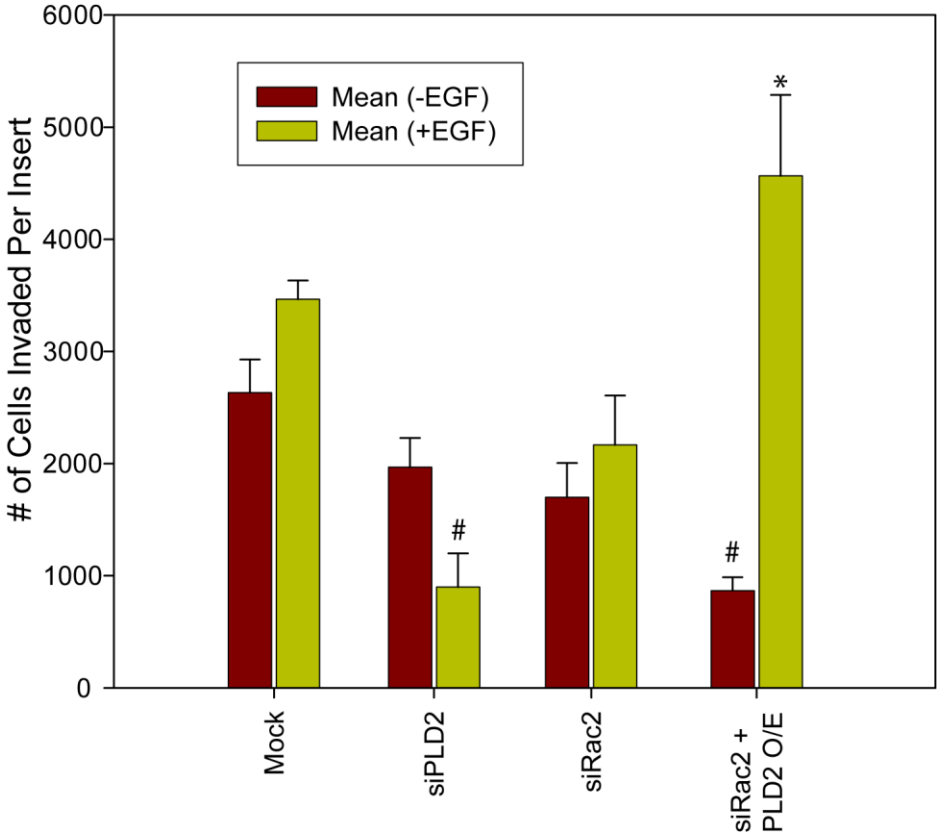


Figure 24:

This silencing cell invasion assay depicted roles for both PLD2 and Rac2-mediated cell invasion in the presence of EGF. Silencing PLD2 resulted in a 70% decrease of cell invasion, while silencing Rac2 resulted in almost a 30% decrease in cell invasion, despite its negative interactions, possibly due to cell invasion functions independent of PLD2. Overexpressing PLD2WT was able to rescue siRac2 and increase cell invasion. With no chemoattractant stimulation, the silenced Rac2 co-transfected with PLD2WT exhibited a decrease in cell invasion by 70%, indicating a possible EGF-mediated interaction between the two proteins, based off the EGF stimulated Rac2 figure shown previously in the introduction of this study.

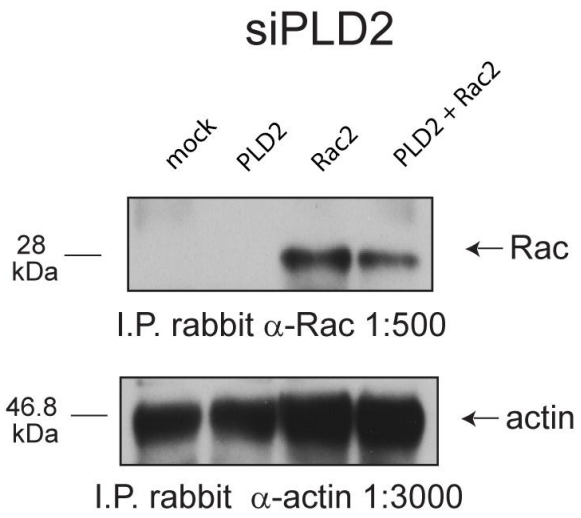


Figure 25:

This IP illustrates the suppression of Rac2 protein expression when PLD2WT is overexpressed. The negative effect of Rac2 on PLD2 may be attributed to protein expression, when PLD2 is silenced Rac2 WT is strongly overexpressed. When PLD2 WT is overexpressed to overcome the siPLD2, Rac2 protein expression diminishes, possibly due to the competitive nature between these two proteins. For PLD2 to mediate cell invasion, it must downregulate Rac2 to negate its effect.

F. Effect of Inhibitors on Rac2-Mediated Cell Invasion and PLD Enzyme Activity

To determine the effect of the 5 inhibitors on Rac2-mediated cell invasion and endogenous PLD lipase activity, we performed a cell invasion assay with PLD2 WT, Rac2 WT and PLD2 WT + Rac2 WT (Figure 26), and these same conditions for an enzyme activity assay (Figure 27). In cell invasion, 40-75% inhibition is exhibited in response to these inhibitors. Our enzyme activity assay, however, does not exhibit the same effect from our inhibitors. Both Rac2 alone and Rac2 + PLD2 transfectants when used to determine lipase activity were consistently resistant to inhibition by these compounds, except for FIPI, which did slightly negatively impact lipase activity of these 2 transfectants. The capability of the compounds to inhibit Rac2 WT and co-transfected Rac2 WT + PLD2 WT in cell invasion and not in enzyme activity suggests Api, 5WO, 809 and APV operate independent of PLD. This PLD independent mechanism is having off-target effects on other proteins and pathways, allowing for inhibition in cell invasion but not on endogenous lipase activity, affecting functions not specific to PLD.

G. CRIB-Mediated Cell Invasion and PLD Enzyme Activity

The PH domain of PLD2 is from amino acid 210-313. CRIB1 is located entirely within the PH domain of PLD2 (I255-HGV280), while CRIB2 extends slightly beyond the PH domain (I306-H323). Because CRIB is the domain on PLD2 where Rac2 binds, partial deletions of this domain were made to observe the effect it has on Rac2 binding to PLD2. Overexpression of these mutants were shown in Western Blot (Figure 28a) and assayed in cell invasion (Figure 28b). ΔCRIB1 has an insignificant effect on cell invasion while ΔCRIB2 has no effect. In the case of cell invasion, our CRIB mutants appear to

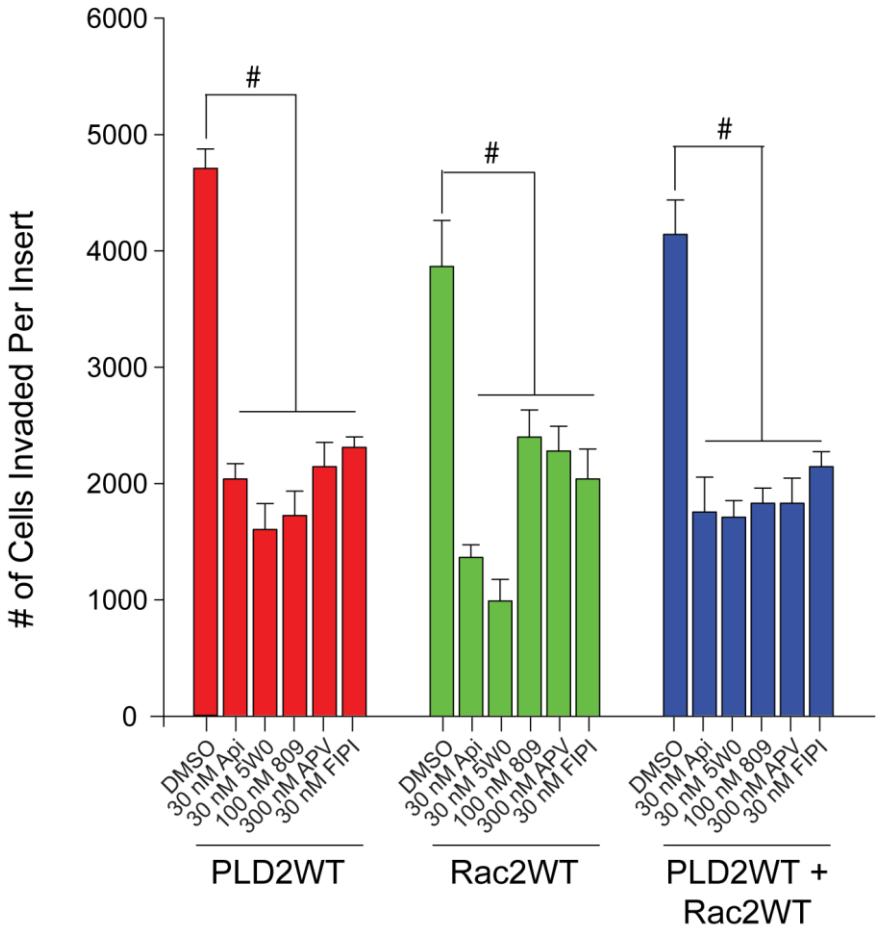


Figure 26:

PLD2 WT is used as a positive control. Cell invasion with overexpressed Rac2 WT are inhibited by our compounds of interest. 809, APV and FIPI inhibit cell invasion by 40% in the Rac2 WT sample, Api and 5WO by 65%. With our co-transfected PLD2 WT + Rac2 WT, cell invasion is inhibited evenly by 50%.

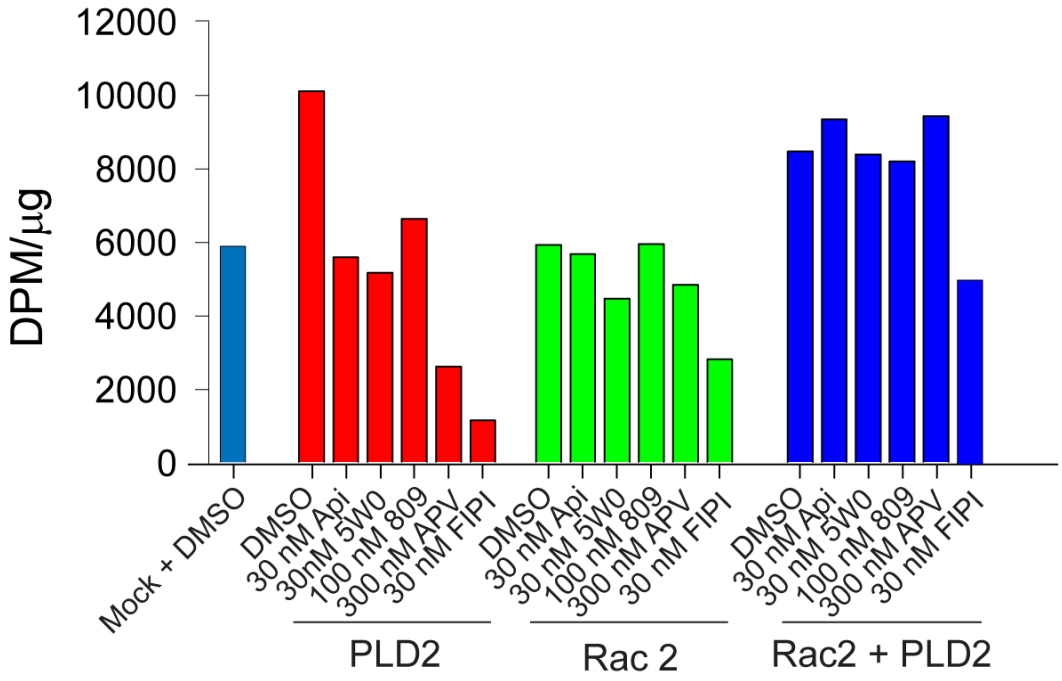


Figure 27:

The mock sample with DMSO is used as a negative control and PLD2 WT is once again the positive control. The effect of Rac2 WT on endogenous PLD lipase activity (green bars) is inhibited slightly by 5WO and APV at 15%, FIPI at 50%, and no inhibition is observed with Api and 809. Co-transfected Rac2 WT + PLD2 WT were only inhibited by FIPI (40%).

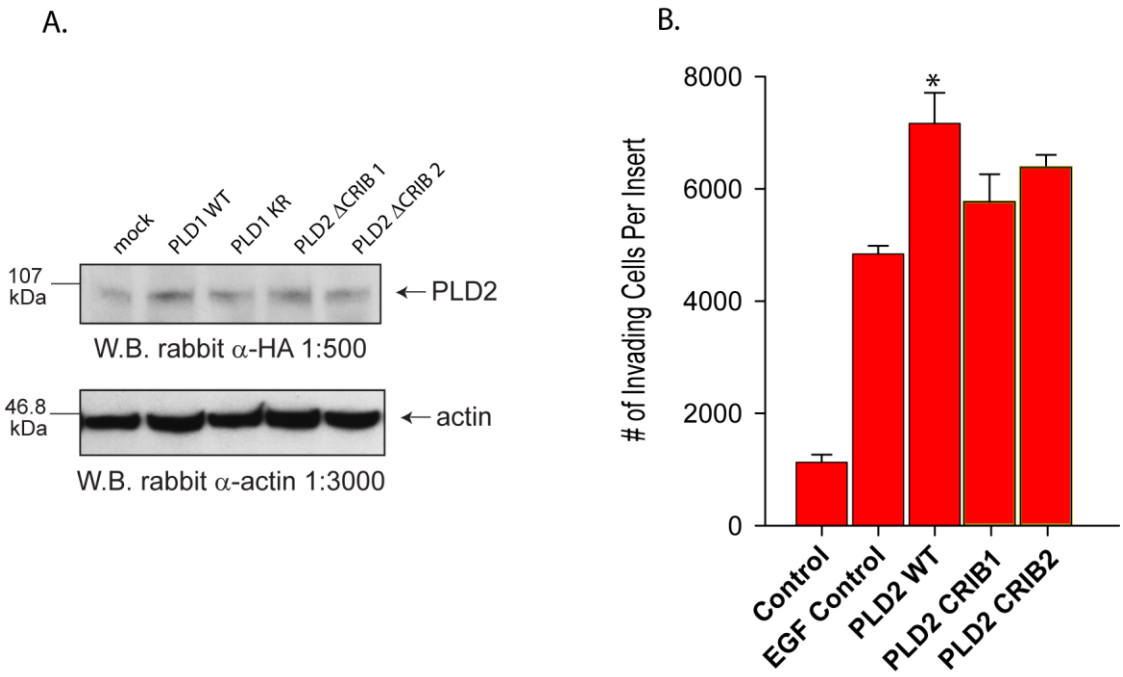


Figure 28:

Part A is a Western Blot of the overexpressed ΔCRIB mutants (PLD1 WT and KR data not shown). Part B illustrates the negative effects on cell invasion by overexpressed ΔCRIB mutants when compared to the PLD2 WT positive control. Although above the EGF control, the ΔCRIB mutants are within a PLD2 construct, only by enhancing cell invasion beyond our PLD2 WT would these mutants have positive effects.

compensate for each other. When ΔCRIB1 is overexpressed, Rac2 may still partially bind to PLD2 via the CRIB2 domain, and vice versa. This compensatory effect was reaffirmed by PLD enzyme activity (Figure 29). With ΔCRIB1 and ΔCRIB2 overexpressed, PLD activity is lower than overexpressed PLD2 WT. This effect is consistent with the PLD lipase activity when Rac2 was transfected into MTLn3 cells. The specificity of Rac2 binding to the CRIB domain is inconsequential in this cell line, as neither deletion was able to completely deter Rac2 binding to PLD2.

H. Effect of Inhibitors on CRIB-Mediated Cell Invasion and PLD Enzyme Activity

The overexpressed CRIB mutants were then exposed to the 5 inhibitors in a cell invasion assay (Figure 30a). Api and FIPI inhibit cell invasion on both CRIB mutants by 55% and 70%, respectively. However, 5WO, 809 and APV inhibit ΔCRIB1 by ~30%, with only 5WO having the same effect on ΔCRIB2. As mentioned earlier, ΔCRIB1 and ΔCRIB2 are compensated by the half of the CRIB domain still intact on the PLD2 molecule. Because Rac2 has no preference for either half of the amino acid residues located within the CRIB domain, the cell invasion results with inhibitors share resemblance in their response to our PLD2 WT controls. In addition to cell invasion, we performed an enzyme activity assay with inhibitors with ΔCRIB2 (Figure 30b).

AIM 4: To Investigate the Effect of Kinase Phosphorylation of PLD2 on PLD2-Mediated Cell Invasion and Lipase Activity.

A. Roadmap of Proteins and Protein Interactions

The fourth and final aim was to investigate the effect of kinases specific to PLD2 (Figure 31). The PLD2 YF mutants replace certain Tyr residues with a Phe residue,

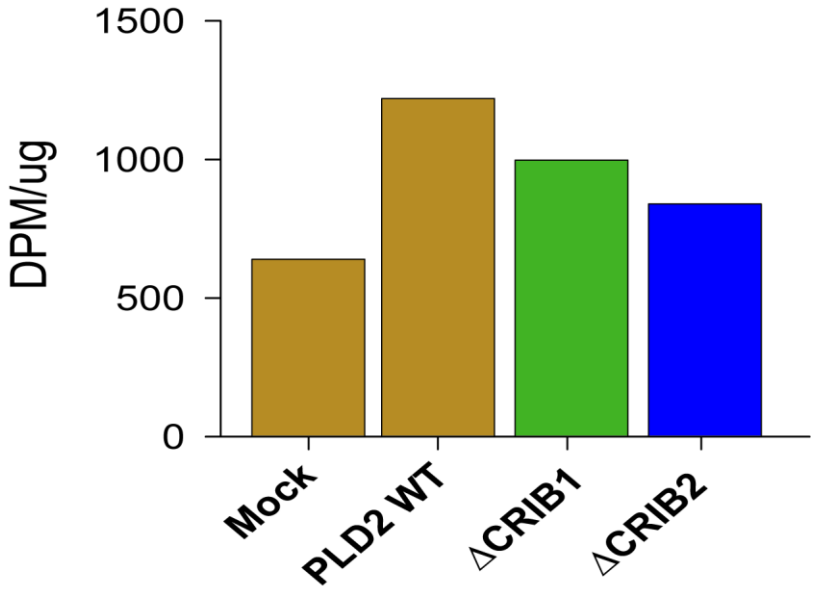


Figure 29:

The enzymatic activity of PLD2 when the CRIB mutants are overexpressed is consistent with the cell invasion data. ΔCRIB1 and ΔCRIB2 are both above the negative (mock) control but below PLD2 WT.

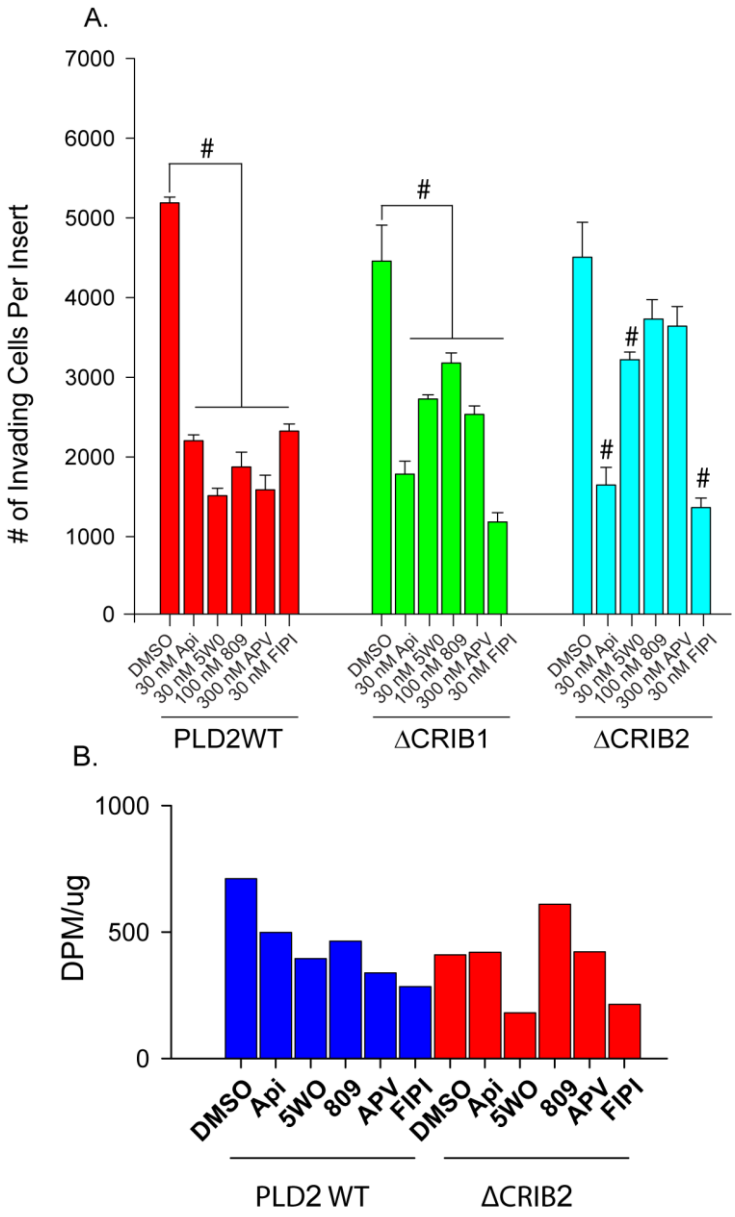


Figure 30:

In Part A, the effect of the inhibitors on cell invasion with overexpressed ΔCRIB mutants is shown. ΔCRIB1 elicits a stronger response from the inhibitors than ΔCRIB2. Part B is the effect of the inhibitors on ΔCRIB2 lipase activity. 5WO and FIPI inhibited enzyme activity by 50%, Api and APV showed no effect, while 809 caused an increase in activity.

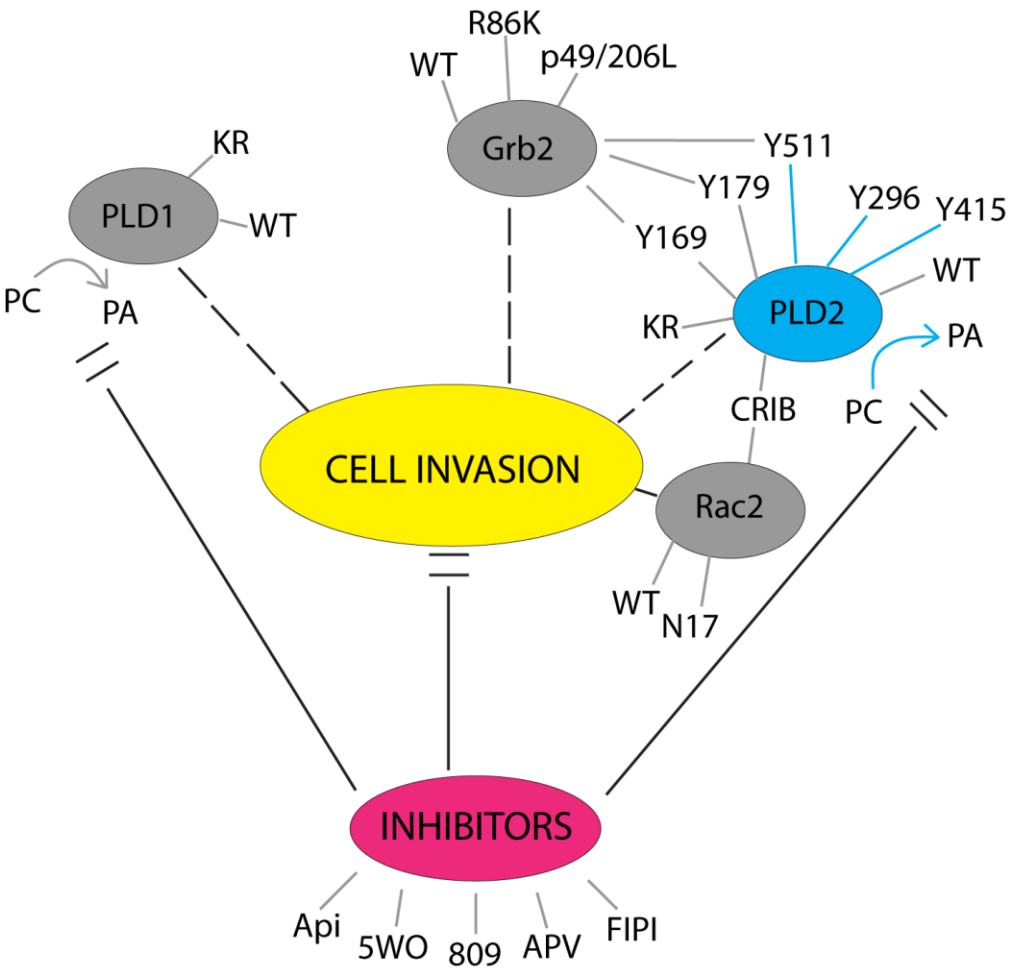


Figure 31:

The final aim of this study is the effect of kinase phosphorylation of PLD2 on PLD2-mediated cell invasion and lipase activity. The Y^{296}, Y^{415} and Y^{511} phosphorylation sites located on PLD2 are all specifically targeted by their respective kinases when regulating PLD2.

eliminating specific kinase phosphorylation sites on PLD2. Each kinase has a specific site that it targets (Y^{296}-EGF-R, Y^{415}-Jak3, Y^{511}-Src), whereby all 3 kinases are regulators of PLD2. Using the mutants of the Y^{296}, Y^{415}, and Y^{511} tyrosyl phosphorylation sites located on PLD2, we defined their effect on cell invasion and enzymatic activity, as well as the effect of our 5 inhibitors on Y296F.

B. Overexpressing Plasmid DNA for Cell Invasion in Matrigels

We overexpressed the YF mutants in a cell invasion assay to observe their effects on cell invasion once kinase phosphorylation on PLD2 is lost (Figure 32). We observed large decreases in cell invasion from the overexpressed Y415F and Y511F mutants, and enhancement of cell invasion with the overexpressed Y296F mutant. Until this point, none of the plasmids when transfected alone enhanced cell invasion beyond PLD2 WT. Because the Y^{296} site is incapable of being phosphorylated by EGF-R, this increase in cell invasion implicates the EGF-R kinase phosphorylation at the Y^{296} site of PLD2 as inhibitory to cell invasion. The dominant negative effects of Y415F and Y511F are due to inactive molecular function, resulting in an antagonistic effect on PLD2.

C. Effect of Inhibitors on Y296F-Mediated Cell Invasion and PLD Enzyme Activity

Due to the emergence of Y296F as a stronger enhancer of cell invasion than PLD2 WT, we assayed the effect of our 5 inhibitors in cell invasion (Figure 33) and lipase activity (Figure 34). These results indicate that the significantly higher lipase activity of Y296F can be inhibited effectively by the 5 compounds.

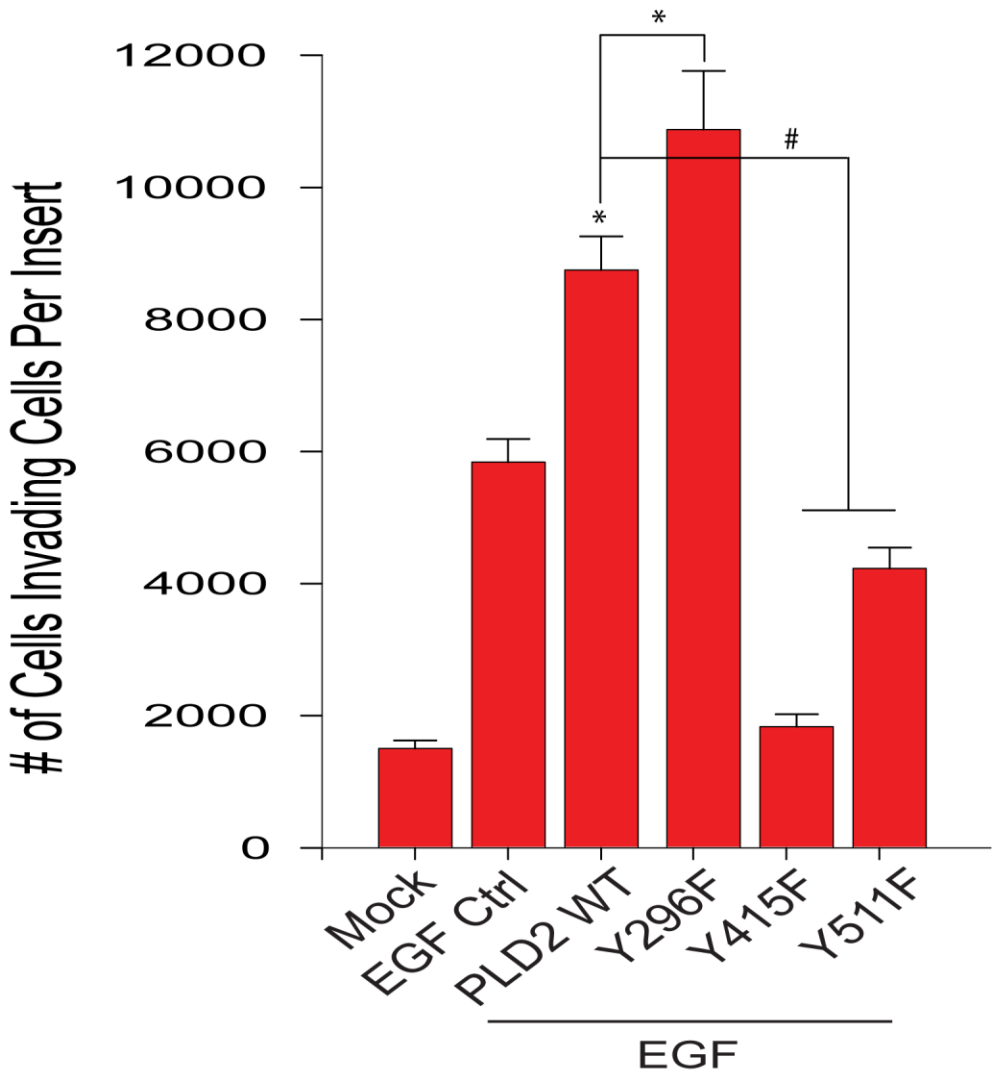


Figure 32:

This cell invasion assay illustrates the effect of the overexpressed PLD2 YF mutants on cell invasion. The Y296F mutant enhances cell invasion above the PLD2 WT positive control by 25%. Our Y415F and Y511F mutants decrease cell invasion by 75% and 50%, respectively. This data indicates that when these tyrosine sites on PLD2 are phosphorylated by their respective kinases, Y^{415} and Y^{511} are activator sites while Y^{296} is inhibitory.

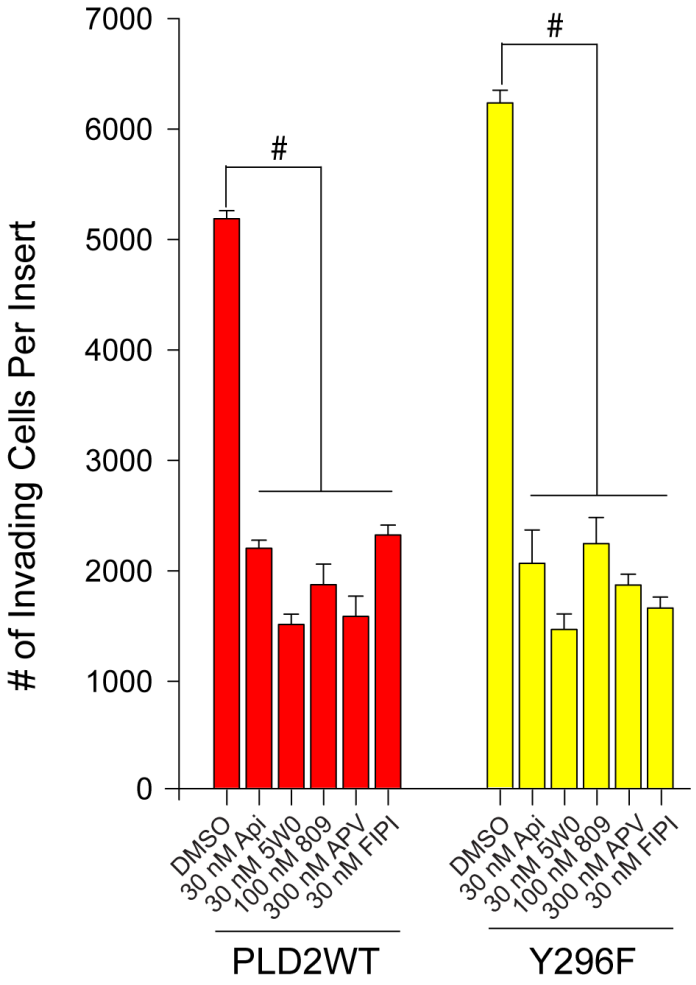


Figure 33:

PLD2 WT is used a positive control. The enhancing effect in cell invasion by Y296F was able to be inhibited significantly. Api and 809 decreased cell invasion by 65%, 5WO by 75% and APV and FIPI by 70%.

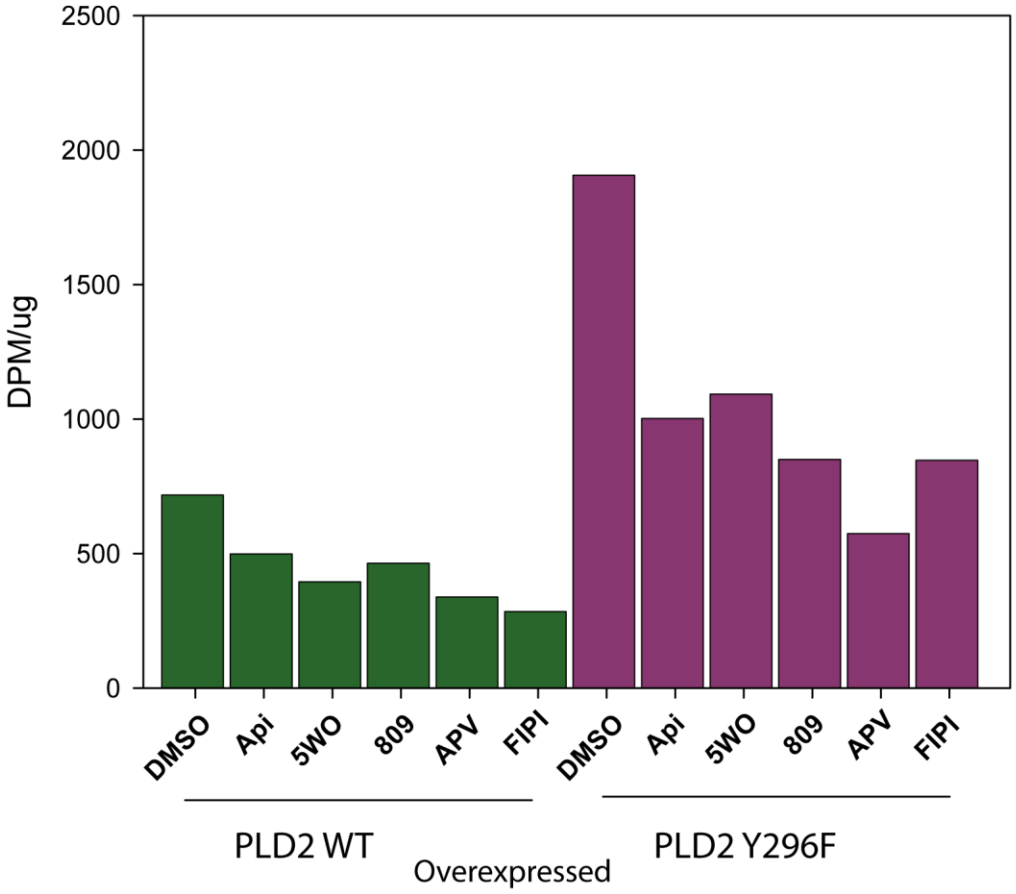


Figure 34:

This PLD enzymatic assay illustrates the 3-fold increase of catalytic activity that Y296F has over the PLD2 WT positive control, consistent with its enhancing effect on cell invasion. Our 5 compounds strongly inhibited catalytic activity, Api and 5WO by 50%, 809 and FIPI by 60% and APV by 70%.

VI. Conclusions

1. PLD2 WT promotes cell invasion. PLD1 WT increases invasion above the EGF control, however, it is below the PLD2 WT positive control, therefore not as potent an enhancer of cell invasion. Both PLD KR mutants significantly decrease cell invasion.

2. Silencing PLD1 was unsuccessful; the silencing of PLD2 resulted in decreased protein expression and lipase activity resulting in an 85% decrease of cell invasion when compared to the EGF and negative siRNA control. With no EGF stimulation, siPLD2 decreased cell invasion by 50% compared to the negative Mock control.

3. Our 5 potential inhibitors targeted and inhibited PLD2 cell invasion and lipase activity significantly (~50% decrease) at subnanomolar concentrations; inhibition of PLD1 was not achieved.

4. Grb2 WT had a positive effect on cell invasion compared to the EGF control, but was not an enhancer of cell invasion when compared to PLD2 WT. The SH2 domain deficient Grb2 R86K mutant had no effect on cell invasion compared to the EGF control and was significantly below that of the positive control. SH3 domain deficient Grb2 p49/206L was strongly inhibitory on cell invasion compared to both controls. When these 3 plasmids were co-transfected with PLD2 WT: Grb2 WT + PLD2 WT was an enhancer of cell invasion, Grb2 R86K + PLD2 WT performed the same as PLD2 WT only and Grb2 p49/206L + PLD2 WT was largely inhibitory when compared to the positive control.

5. Overexpression of PLD2 mutants Y169F, Y179F and Y511F all significantly decreased cell invasion between 60-90%.

6. Silencing Grb2 decreased cell invasion by 75%, although the effects were less than that of siPLD2. siGrb2 + siPLD2 co-silencing almost completely abrogated cell invasion.

7. Silencing PLD2 has no effect on Grb2 protein expression.

8. The 5 compounds successfully inhibited Grb2 WT and co-transfected Grb2 WT + PLD2 WT in cell invasion. Api, 5WO, 809 and APV decreased cell invasion by 60%. FIPI emerged as the strongest inhibitor in the co-transfected sample, decreasing cell invasion by 70%

9. FIPI was capable of inhibiting the lipase activity of PLD2 WT by up to 90%.

10. Rac2 WT has a neutral to negative effect on cell invasion. Rac2 N17 increases cell invasion above that of the EGF control but less so than the PLD2 WT positive control, indicating a positive response when Rac2 is constitutively GDP-bound.

11. Rac2 has a more pronounced negative effect when interacting with PLD2. When Rac2 WT was simultaneously transfected with PLD2 WT cell invasion largely decreased, when sequentially transfected before PLD2 WT, cell invasion was again decreased. When the sequential transfection was inversed, cell invasion was actually enhanced.

12. The results of cell invasion of Rac2 + PLD2 are consistent with lipase activity when co-transfected, recombinant PLD2 enzymatic activity is decreased to that of endogenous levels.

13. Silencing Rac2 resulted in a 30% decrease in cell invasion, indicating a potential role in cell invasion independent of PLD2. Overexpressing PLD2 WT was able to rescue siRac2 and increase cell invasion.

14. With no EGF stimulation, siRac2 + PLD2 WT exhibited a 70% decrease in cell invasion, while stimulation with EGF yielded a 40% increase in cell invasion. This implicates an EGF-mediated interaction between the two proteins.

15. Immunoprecipitation revealed Rac2 protein expression to be suppressed when PLD2 WT is overexpressed.

16. Rac2 WT overexpression in cell invasion was inhibited by the compounds: 809, APV and FIPI by 40%, Api and 5WO by 65%. Rac2 WT + PLD2 WT were inhibited evenly by 50% when any of the 5 compounds were present.

17. The effect of Rac2 WT on endogenous PLD was only partially inhibited by the compounds: 5WO and APV by 15%, FIPI at 50% and no inhibition with Api or 809. Rac2 WT + PLD2 WT was only inhibited by FIPI (40%).

18. Compared to the positive control, ΔCRIB1 had a negative effect on cell invasion, while ΔCRIB2 was slightly more neutral.

19. ΔCRIB1 and ΔCRIB2 decreased endogenous levels of PLD activity when overexpressed, consistent with cell invasion results.

20. In regards to cell invasion and lipase activity, the specificity of Rac2 binding to the CRIB domain is inconsequential in this cell line, as neither deletion was able to completely deter Rac2 binding to PLD2.

21. ΔCRIB1 and ΔCRIB2 were inhibited similarly by the compounds in cell invasion: ΔCRIB1 and ΔCRIB2 with Api by 55% and FIPI by 70%, 5WO, 809, and APV inhibited ΔCRIB1 by 30%, while only 5WO had the same effect on ΔCRIB2. In PLD enzyme activity: 5WO and FIPI inhibited lipase activity by 50%, with no effect from Api or APV and a spike in activity with 809.

22. Y415F and Y511F exhibited large negative effects on cell invasion, while Y296F emerged as the sole overexpressed plasmid to enhance cell invasion.

23. The lipase activity of Y296F yielded a 3-fold increase over PLD2 WT

24. Inhibition of Y296F by the compounds in both cell invasion and lipase activity were very similar in effect and magnitude to PLD2 WT. In cell invasion, Api and 809 decreased cell invasion by 65%, 5WO by 75% and APV and FIPI by 70%. The compounds inhibited lipase activity effectively: Api and 5WO by 50%, FIPI by 60% and APV by 70%.

VII. Discussion

PLD2-mediated cell invasion

The results confirmed that PLD2 mediates cell invasion in the MTLn3 cell line when stimulated by EGF (Figure 35). The consistent enhancement of cell invasion when PLD2 was the sole transfectant was 40% above the EGF control on average. Additionally, the silencing of PLD2 decreased cell invasion between 70-85%.

Protein-Protein Interaction with Grb2

Grb2 WT does not enhance cell invasion nor increase endogenous PLD activity. However, the silencing of Grb2 resulted in a 75% decrease in cell invasion and the co-silencing of Grb2 + PLD2 almost completely abrogates it. When Grb2 is co-transfected with PLD2, cell invasion and lipase activity were slightly enhanced, indicating a role for Grb2 as a complementary partner in PLD2-mediated cell invasion. The PLD2 YF and Grb2 mutants that eliminate the interaction of PLD2 with Grb2 have significantly negative effects on cell invasion. When compared to PLD2 WT, Grb2 R86K was 30% decreased, Grb2 p49/206L and Y511F decreased 60%, Y169F was decreased 70% and Y179F was decreased 80%. Grb2 is incapable of interacting with PLD2 when the R86K mutant is overexpressed due to the loss of the SH2 domain [21]. The SH3 domain-deficiency of the Grb2 p49/206L double mutant when overexpressed results in the loss of the complex formation with the Ras guanine nucleotide exchange factor Sos [63,90]. PLD2 residues Y^{169} and Y^{179} bind to Grb2, modulate activity and regulate tyrosine phosphorylation, respectively [21]. Both of these tyrosine residues are necessary for the recruitment of Sos. MTLn3 cells are less invasive when Grb2 cannot bind to PLD2, but

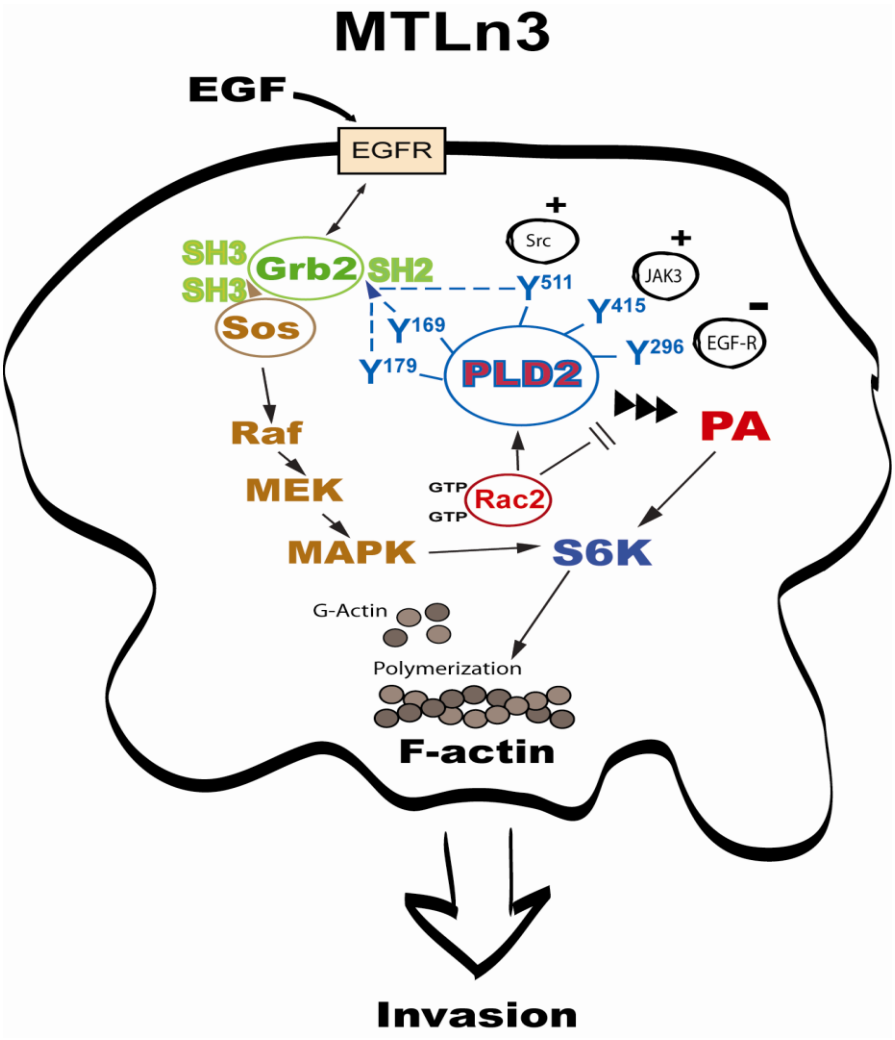


Figure 35:

The interaction between Grb2 and PLD2 through specific tyrosine residues on PLD2 target the SH2 domain of Grb2, recruiting Sos to the SH3 domain of Grb2 in a trimeric complex. The PLD2 tyrosine specific kinases act upon their respective sites. The Y^{511} site is involved in both functions, however its ability to bind to Grb2 is more limited, as its main function is as a kinase phosphorylation site. GTP-bound Rac2 WT binds to PLD2 on the CRIB domain, negatively affecting catalytic activity. When sufficient amounts of PA are produced within the cell, S6K is phosphorylated to mediate actin polymerization at the leading edge of the cell, resulting in invasion [91].

even less so when PLD2 cannot interact with the Grb2/Sos complex to promote GTP loading of Ras. Ras effectors are no longer activated, nor are the Ras/MAPK pathways downstream, resulting in an inhibitory effect on cell invasion. Ho et. al have shown that inhibition of cell invasion and migration can be achieved by suppressing the MAPK pathway through inhibition of Grb2 (among others such as Ras, Rho and ERK)[92], which indicates the PLD2/Grb2 interaction as a prominent target of inhibition. The interaction between PLD2 and Grb2 is a crucial cellular signaling process within PLD2-mediated pathways and although not a synergistic effect between the two, the presence of both proteins is essential in PLD2-mediated cell invasion.

Protein-Protein Interaction with Rac2

Rac2 WT has a neutral to negative effect on cell invasion when transfected alone, conversely, the mutant GDP-bound Rac2 N17 is constitutively inactive, resulting in an increase in cell invasion. These negative effects become more pronounced with concomitant amounts of Rac2 WT co-transfected with PLD2 WT. When recombinant proteins are co-transfected into MTLn3 cells, Rac2 inhibits both endogenous and overexpressed PLD2 enzymatic activity. Parker et. al have shown that Rac2 is downregulated in ovarian cancer [93], in agreement with our results that Rac2 may possess antagonistic properties in cancer progression. The negative effect of Rac2 on PLD2 was only overcome when PLD2 WT was sequentially transfected before Rac2 WT, allowing a longer time period in which PLD2 can be expressed prior to cell invasion. A schematic from our lab has proposed competition with PIP_2 as the main cause of negative interaction (Figure 36). When silencing Rac2, a 30% decrease in invasion was observed

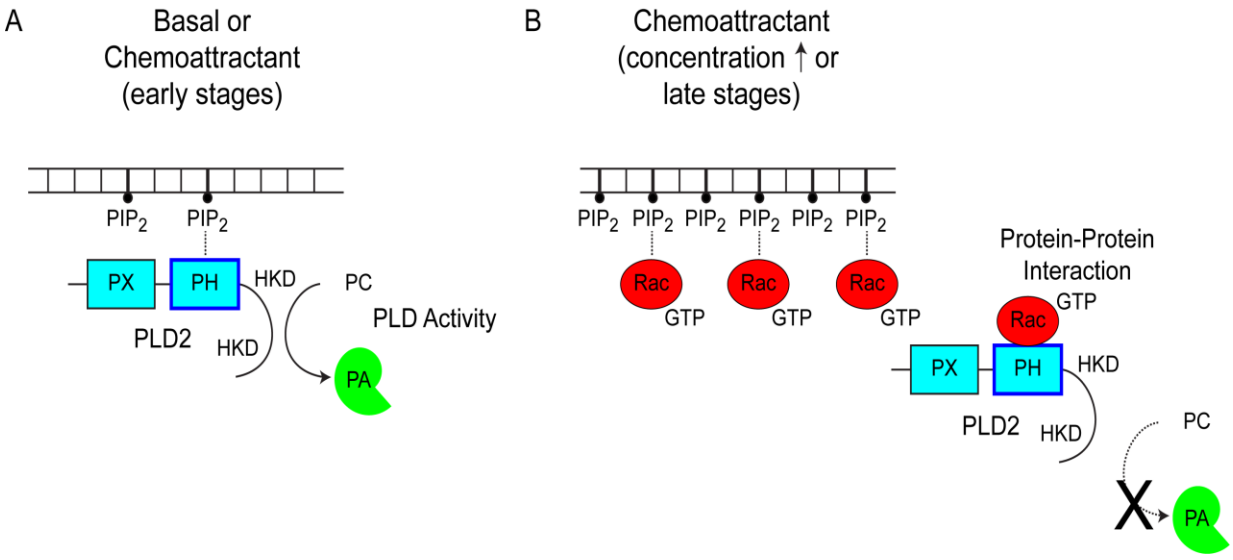


Figure 36:

In part A, at basal levels or early stages of chemoattractant exposure, PLD2 binds to PIP_2 freely and PLD enzymatic activity is unperturbed. When chemoattractant is present in higher amounts, or exposure time is lengthened, the amount of GTP-bound Rac2 present within the cell increases. Competition with PIP_2 available on the cell membrane, as well as the binding of Rac2 to the CRIB domain located on PLD2's PH domain, negatively affects the catalytic activity of PLD2.

in the presence of EGF. When siRac2 + overexpressed PLD2 WT were co-transfected, the negative effect of siRac2 in cell invasion was rescued in the presence of EGF, which resulted in an overall increase in cell invasion when compared to the mock sample. When EGF was not present for this condition, a 70% decrease in cell invasion was observed, indicating that Rac2 has an EGF-mediated effect on PLD2. Therefore, in basal stages or limited exposure to chemoattractant, PLD2 freely binds to PIP_2 without interference from Rac2. When EGF increases in concentration, the inverse effect occurs, in which GTP-bound Rac2 competes with PIP_2 binding on the cell membrane. PLD2, increasingly unable to bind PIP_2, is additionally bound more frequently by Rac2 on its PH domain. This dynamic, in turn, results in a decrease of cellular PA production, decreasing lipase activity and cell invasion.

The protein-protein interaction of Rac2 and PLD2 occurs on the CRIB domain of PLD2 (Figure 37), which was studied through the use of the ΔCRIB1 and ΔCRIB2 mutants. When the ΔCRIB mutants were overexpressed, they were unable to enhance cell invasion. The lipase activity of PLD2 was decreased, as Rac2 was still able to bind to PLD2. These results indicated that the specificity of Rac2 binding to the CRIB domain is inconsequential in this cell line, as neither deletion was able to completely deter Rac2 from binding to PLD2.

The effect of kinase phosphorylation on PLD2

Three sites on PLD2 regulated by kinase phosphorylation were examined through the use of the YF mutants which are incapable of being phosphorylated by certain kinases: Y415F and Y511F decreased cell invasion by almost 80% and 60%,

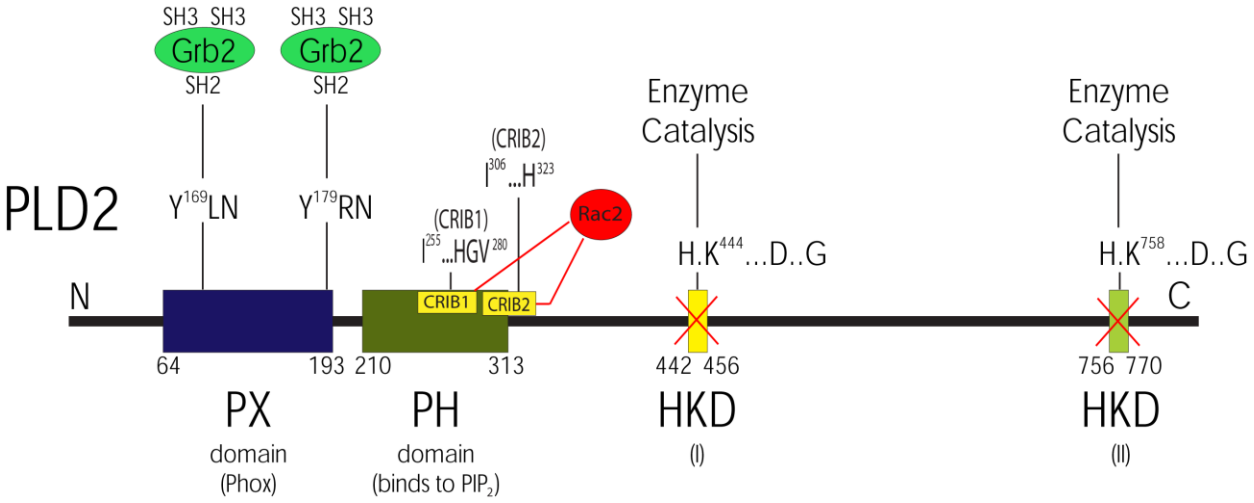


Figure 37:

CRIB1 (I255-HGV280) and CRIB2 (I306-H323) were deleted in our ΔCRIB mutants to counteract the negative effect of Rac2 on PLD2 catalytic activity. These deletions were ineffective in the MTLn3 cell line due to the non-specific nature of Rac2 binding on the CRIB domain.

respectively, while Y296F enhanced cell invasion by 25%. Additionally, Y296F increased the lipase activity of PLD2 3-*fold* over PLD2 WT and was the only plasmid transfected solely that was capable of enhancing both cell invasion and lipase activity. This data indicates that Y^{415} and Y^{511} are activatory sites on PLD2, while Y^{296} is inhibitory. These mutants and their respective kinases (Y^{296}: EGF-R), (Y^{415}: Jak3) and (Y^{511}: Src) were examined in greater detail by our lab (Figure 38). Figure 38a depicts the decrease of PLD2 lipase activity when Y415F and Y511F are overexpressed. Our second figure, 38b, is the result of incubating our Y296F plasmid with the kinases EGF-R, Jak3 and Src to determine their effect on Y296F lipase activity. Because the Y296 site is no longer available for phosphorylation, EGF-R has no effect, while Jak3 and Src increase lipase activity by 40% and 45%, respectively. The kinases Jak3 and Src are activators of PLD2, which compensate for the inhibitory effect of EGF-R. Henkels et. al have shown the Y^{296} site as inhibitory in MCF-7 cells, a low-invasive breast cancer cell line that exhibits little lipase activity [37]. MTLn3 cells have medium to low lipase activity [37] and both are estrogen receptor positive. This places MTLn3 cells as an intermediate between high lipase and low lipase expressing breast cancers, indicating catalytic activity in these cells as one factor, while reaffirming the importance of the protein-protein interactions and their mitogenic signaling effects in the PLD pathway.

The capability of PLD2 overexpression to increase cell growth and induce cell transformation has been well characterized by the lab of David Foster [94,95] and Henkels et.al have shown that when PLD2 lipase activity is low due to PLD2 phosphorylation deficiency, cellular proliferation is decreased [66], both results in accordance concerning the consequence of PLD2 and its lipase activity. The compensatory effects of the Jak3

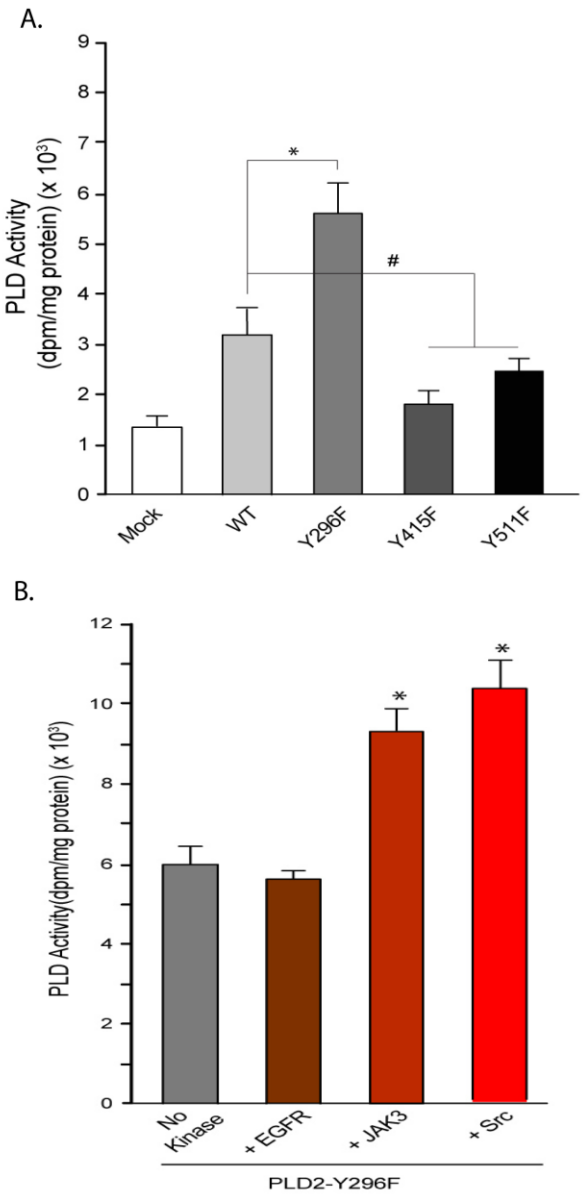


Figure 38:

Both figures utilize the MTLn3 cell line. Part A depicts the decrease in PLD enzyme activity when Y415F and Y511F are overexpressed, and the enhancing effect when Y296F is overexpressed. Part B depicts the effect of Y296F on enzyme activity, which is increased by Jak3 and Src kinases, while EGF-R has no effect, as the Y^{296} site is no longer available for phosphorylation [37].

and Src kinases are utterly crucial for PLD2-mediated cell invasion; therefore, the knockdown of these two specific kinases would greatly decrease the invasive capabilities of MTLn3 cells undergoing metastasis.

The inhibitory effect of our 5 potential inhibitors

The 5 potential inhibitors used in this study were Apigenin, 5WO, 809, APV and FIPI. While Apigenin and FIPI have been characterized by others, 5WO, 809 and APV are still relatively uncharacterized. Frohman's lab has shown that FIPI specifically targets PLD [84], with inhibitory results on PLD2 activity similar to those found in this study. The other 4 compounds, Api, 5WO, 809 and APV have been proposed to also have an affinity for targeting PLD. All 5 compounds were able to inhibit PLD2 WT cell invasion and lipase activity by at least 50%. However, when the Grb2/PLD2 and Rac2/PLD2 protein-protein interactions were elucidated, FIPI emerged as the strongest and most consistent inhibitor in both cell invasion and lipase activity. Apigenin was the least effective, while 5WO, 809 and APV all had middling effects on inhibiting cell invasion and lipase activity, indicating these 4 compounds operate independent of PLD. When PLD2 WT + Grb2 WT were co-transfected, FIPI inhibited cell invasion and lipase activity by 70%. We also observed up to 90% inhibition on overexpressed PLD2 WT lipase activity when FIPI was present. When looking at Rac2, cell invasion was consistently inhibited across the board; however, the effect of Rac2 on endogenous PLD was only significantly inhibited by FIPI (50%), as well as the lipase activity of co-transfected Rac2 WT + PLD2 WT (40%). FIPI was also capable of inhibiting the effect of CRIB2 on lipase activity (50%), as well as Y296F (60%). Inhibition of PLD has been described using neomycin [96],

ceramide [97] and natural products (such as Honokiol), a compound that suppresses tumor growth in mice and has been shown to suppress PLD activity in human cancer cells [98]. These compounds either sequester the requisite PLD cofactor PIP_2, work indirectly to inhibit PLD activity, or have effects independent from the PLD signaling pathway. 5WO, 809 and APV are compounds operating through undetermined mechanisms, their inhibition on PLD shown in this study are indirect effects not specific to PLD. The results show inhibition of PLD2-mediated cell invasion and lipase activity, but inadequate inhibition on Rac2 and Grb2's effects on endogenous lipase activity. This confirms 5WO, 809 and APV are only indirectly inhibiting PLD. The decrease in inhibition when protein-protein interactions are involved might be due to the fact that the compounds effect downstream signaling molecules, or 5WO, 809 and APV bind to the same sites on PLD where PLD-associated proteins like Grb2 and Rac2 bind. Apigenin has many known mechanisms through which it inhibits, and has shown promise in inhibiting tumor cell invasion and metastases through regulation of protease production [85], the results from this study; however, show no specific effect for PLD. Again, the protein-protein interactions revealed in this study show the effect of Api on PLD2-mediated cell invasion and lipase activity are indirect, due to Api's inhibitory effects on other proteins and signaling pathways.

Api, 5WO, 809 and APV all provided inhibitory effects, although independent of PLD, indicating promising therapeutic utilization on other proteins and signaling pathways. Based on the results of this study, it is evident that the inhibition of PA by FIPI [84] is the most prevalent way to target and decrease PLD-mediated cell invasion and lipase activity. The potent and specific effects of FIPI have proved to be the most effective at

decreasing PLD-mediated cell invasion. MTLn3, as an optimal model of breast cancer, necessitates PLD2-mediation for efficient intravasation, in which FIPI can interrupt to deter metastatic progression.

Therapeutic Approach

We propose here, that a therapeutical approach could be made possible that would inhibit the crucial step of PLD2-mediated cell invasion during metastasis in MTLn3 cells. The ability of FIPI to inhibit PLD2-mediated cell invasion and lipase activity is the reason for its inclusion as one part of our therapeutic approach. The other part of this approach is focused upon PLD2 regulation by kinase phosphorylation, in which we know that the kinases Jak3 and Src can phosphorylate PLD2 and increase its lipase activity. Protein-protein interactions with PLD2 indicate that in the presence of EGF, Rac2 is inherently acting against cell invasion through a negative catalytic interaction, while Grb2 serves as a partner for PLD2 to activate cellular functions congruent with cell invasion. PLD lipase activity can be increased up to 3-*fold* above normal when EGF-R kinase phosphorylation is lost, indicating a second protein that can negatively interact with PLD2. As the EGF-R kinase negatively regulates PLD2, knocking down the activator kinases Jak3 and Src, which compensate for PLD2 activity, should provide a lower threshold in which PLD2 can mediate cell invasion. This knockdown of the Jak3 and Src kinases, in combination with utilization of the small molecule inhibitor FIPI to inhibit PA production, may result in the inhibition of PLD2-mediated cell invasion to near basal levels in spite of stimulation by EGF. The result of lower PLD2 lipase activity,

in conjunction with decreased cellular PA production, should protect against the numerous invasive and metastatic cellular functions active within this pathway.

VIII. Significance of Study

Cell invasion is a crucial component of angiogenesis and metastasis and often targeted in cancer therapy. The prowess of PLD to mediate cell invasion is well characterized, and specific interactions with other proteins involved in cancer progression were further examined here. The effects on cell signaling by Grb2 and Rac2 in MTLn3 cells give us further insight into the mechanisms regulating this adenocarcinoma, including one of the foremost examples of the Rac2/PLD2 interaction in cancer. Our therapeutical approach presents a strong opportunity to inhibit tumor progression not only in MTLn3s, but possibly other cancer models related to metastasis.

IX. References

1. Pantel K BR. Dissecting the metastatic cascade. Nat Rev Cancer 2004;4:448-456.
2. Ferlay J AP, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007;18:581-592.
3. Marxfeld H SF, Harleman JH. Characterisation of two rat mammary tumour models for breast cancer research by gene expression profiling. exp toxicol pathol 2006;58:133-143.
4. Xue C WJ, Liang F, Siadni M, Violine S, Tasi KL, Zhang ZY, Sahai E, Condeelis J, Segall J. Epidermal growth factor receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. cancer res 2006;66:192-197.
5. Cockcroft S. Signalling roles of mammalian phospholipase D1 and D2. Cell Mol Life Sci 2001;58(11):1674-87.
6. Yang SF, Freer, S., Benson, A.A. Transphosphatidylation by phospholipase D. Journal of Biological Chemistry 1967;242:477-484.
7. Hanahan DJ CI. The phosphorous-containing lipids of the carrot. J biol chem 1947;168:233-240.
8. Hanahan DJ CI. On the nature of the phosphorous-containing lipids of cabbage leaves and their relation to phospholipids-splitting enzyme contained in these leaves. j biol chem 1948;172:191-198.
9. Liscovitch M, Czarny M, Fiucci G, Tang X. Phospholipase D: molecular and cell biology of a novel gene family. Biochem J 2000;345 Pt 3:401-15.
10. Liscovitch MC, L.C. Lipid Second Messengers. Cell 1994;77:329-334.

11. Exton JH. Phospholipase D: enzymology, mechanisms of regulation, and function. Physiol Rev 1997;77(2):303-20.

12. Freyberg Z, Siddhanta A, Shields D. "Slip, sliding away": phospholipase D and the Golgi apparatus. Trends Cell Biol 2003;13(10):540-6.

13. Park SH, Chun YH, Ryu SH, Suh PG, Kim H. Assignment of human PLD1 to human chromosome band 3q26 by fluorescence in situ hybridization. Cytogenet Cell Genet 1998;82(3-4):224.

14. Park SH, Ryu SH, Suh PG, Kim H. Assignment of human PLD2 to chromosome band 17p13.1 by fluorescence in situ hybridization. Cytogenet Cell Genet 1998;82(3-4):225.

15. Kook S, Exton JH. Identification of interaction sites of protein kinase Calpha on phospholipase D1. Cell Signal 2005;17(11):1423-32.

16. Choi WS, Hiragun T, Lee JH, Kim YM, Kim HP, Chahdi A, Her E, Han JW, Beaven MA. Activation of RBL-2H3 mast cells is dependent on tyrosine phosphorylation of phospholipase D2 by Fyn and Fgr. Mol Cell Biol 2004;24(16):6980-92.

17. Steed PM, Clark KL, Boyar WC, Lasala DJ. Characterization of human PLD2 and the analysis of PLD isoform splice variants. Faseb J 1998;12(13):1309-17.

18. Henage LG, Exton JH, Brown HA. Kinetic analysis of a mammalian phospholipase D: allosteric modulation by monomeric GTPases, protein kinase C, and polyphosphoinositides. J Biol Chem 2006;281(6):3408-17.

19. Sciorra VA, Rudge SA, Prestwich GD, Frohman MA, Engebrecht J, Morris AJ. Identification of a phosphoinositide binding motif that mediates activation of

mammalian and yeast phospholipase D isoenzymes. Embo J 1999;18(21):5911-21.

20. Sciorra VA, Rudge SA, Wang J, McLaughlin S, Engebrecht J, Morris AJ. Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J Cell Biol 2002;159(6):1039-49.

21. Di Fulvio M, Lehman N, Lin X, Lopez I, Gomez-Cambronero J. The elucidation of novel SH2 binding sites on PLD2. Oncogene 2006;25(21):3032-40.

22. Lee CS, Kim IS, Park JB, Lee MN, Lee HY, Suh PG, Ryu SH. The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis. Nat Cell Biol 2006;8(5):477-84.

23. Noh DY, Ahn SJ, Lee RA, Park IA, Kim JH, Suh PG, Ryu SH, Lee KH, Han JS. Overexpression of phospholipase D1 in human breast cancer tissues. Cancer Lett 2000;161(2):207-14.

24. Uchida N, Okamura S, Nagamachi Y, Yamashita S. Increased phospholipase D activity in human breast cancer. J Cancer Res Clin Oncol 1997;123(5):280-5.

25. Uchida N, Okamura S, Kuwano H. Phospholipase D activity in human gastric carcinoma. Anticancer Res 1999;19(1B):671-5.

26. Zhao Y, Ehara H, Akao Y, Shamoto M, Nakagawa Y, Banno Y, Deguchi T, Ohishi N, Yagi K, Nozawa Y. Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. Biochem Biophys Res Commun 2000;278(1):140-3.

27. Yamada Y, Hamajima N, Kato T, Iwata H, Yamamura Y, Shinoda M, Suyama M, Mitsudomi T, Tajima K, Kusakabe S, Yoshida H, Banno Y, Akao Y, Tanaka M,

Nozawa Y. Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. J Mol Med 2003;81(2):126-31.

28. Foster DA, Xu L. Phospholipase D in cell proliferation and cancer. Mol Cancer Res 2003;1(11):789-800.

29. Freyberg Z, Bourgoin S, Shields D. Phospholipase D2 is localized to the rims of the Golgi apparatus in mammalian cells. Mol Biol Cell 2002;13(11):3930-42.

30. Tanabe K TT, Natsume W, Anderson SB, Watanabe T and Satake M. A Novel GTPase-activating protein for ARF6 Directly Interacts with Clathrin and Regulates Clathrin-dependent Endocytosis. MBoc 2005;16:1617-1628.

31. Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman MA, Kanaho Y. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell 1999;99(5):521-32.

32. Lopez I, Arnold RS, Lambeth JD. Cloning and initial characterization of a human phospholipase D2 (hPLD2). ADP-ribosylation factor regulates hPLD2. J Biol Chem 1998;273(21):12846-52.

33. Siddiqi AR, Srajer GE, Leslie CC. Regulation of human PLD1 and PLD2 by calcium and protein kinase C. Biochim Biophys Acta 2000;1497(1):103-14.

34. Kim JH, Kim Y, Lee SD, Lopez I, Arnold RS, Lambeth JD, Suh PG, Ryu SH. Selective activation of phospholipase D2 by unsaturated fatty acid. FEBS Lett 1999;454(1-2):42-6.

35. Slaaby R, Du G, Altshuller YM, Frohman MA, Seedorf K. Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293

cells mediated by the phospholipase C gamma and protein kinase C alpha signalling cascade. Biochem J 2000;351 Pt 3:613-9.

36. Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell 1993;75(6):1137-44.

37. Henkels KM PH, Frondorf K, Gomez-Cambronero J. A comprehensive model that explains the regulation of phospholipase D2 activity by phosphorylation-dephosphorylation. Mol Cell Biol 2010;30:2251-2263.

38. David AF LX. Phospholipase D in Cell Proliferation and Cancer. Mol Cancer Res 2003;1:789-800.

39. Cross MJ, Roberts S, Ridley AJ, Hodgkin MN, Stewart A, Claesson-Welsh L, Wakelam MJ. Stimulation of actin stress fibre formation mediated by activation of phospholipase D. Curr Biol 1996;6(5):588-97.

40. Bar-Sagi TJaD. Ras effectors and their role in mitogenesis and oncogenesis. Journal of Molecular Medicine 1997;75:587-593.

41. Vogt A, Pestell KE, Day BW, Lazo JS, Wipf P. The antisignaling agent SC-alpha alpha delta 9, 4-(benzyl-(2-[(2,5-diphenyloxazole-4-carbonyl)amino]ethyl)carbamoyl)- 2-decanoylaminobutyric acid, is a structurally unique phospholipid analogue with phospholipase C inhibitory activity. Mol Cancer Ther 2002;1(11):885-92.

42. Bonnans C LB. Lipid mediators as agonists for the resolution of acute lung inflammation and injury. Am J Respir Cell Mol Biol 2007;36:201-205.

43. Li P, Zhou HL, Chen JQ. [Phospholipase D and inflammation]. Sheng Li Ke Xue Jin Zhan 1999;30(2):118-22.

44. Iyer SS, Barton JA, Bourgoin S, Kusner DJ. Phospholipases D1 and D2 coordinately regulate macrophage phagocytosis. J Immunol 2004;173(4):2615-23.

45. Frondorf K, Henkels KM, Frohman MA, Gomez-Cambronero J. Phosphatidic acid (PA) is a leukocyte chemoattractant that acts through S6 kinase signaling. J Biol Chem 2010;285:15837-15847.

46. Ghosh S. SJC, Sciorra V.A., Daniel L. And Bell R.M. Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. J Biol Chem 1996;271:8472-8480.

47. Rizzo MA, Shome K, Vasudevan C, Stolz DB, Sung TC, Frohman MA, Watkins SC, Romero G. Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. J Biol Chem 1999;274(2):1131-9.

48. Ktistakis CLSaNT. Phosphatidic acid and phosphatidylserine-binding proteins. Biochim Biophys Acta - Molecular and Cell Biology of Lipids 2006;1761:913-926.

49. Raghu P, Manifava M, Coadwell J, Ktistakis NT. Emerging findings from studies of phospholipase D in model organisms (and a short update on phosphatidic acid effectors). Biochim Biophys Acta 2009;1791(9):889-97.

50. Schmelzle TaH, M.N. mTOR, a central controller of cell growth. Cell 2000;103:253-262.

51. Gingras AC RB, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes Dev 2001;15:807-826.

52. Volvarevic S SM, Ledermann B, Zilberman F, Terracciano L, Montini E, Grompe M, Kozma SC, Thomas G. Proliferation, but not growth, blocked by conditional deletion of 40s ribosomal protein S6. Science 2000;288:2045-2047.

53. Lehman N, Ledford B, Di Fulvio M, Frondorf K, McPhail LC, Gomez-Cambronero J. Phospholipase D2-derived phosphatidic acid binds to and activates ribosomal p70 S6 kinase independently of mTOR. Faseb J 2007;21(4):1075-87.

54. Kerkhoff EaRUR. High-intensity Raf signals convert mitotic cell cycling into cellular growth. Cancer Res 1998;58:1636-1640.

55. Zhong M, Shen Y, Zheng Y, Joseph T, Jackson D, Foster DA. Phospholipase D prevents apoptosis in v-Src-transformed rat fibroblasts and MDA-MB-231 breast cancer cells. Biochem Biophys Res Commun 2003;302(3):615-9.

56. Hanahan D WR. The hallmarks of cancer. Cell 2000;100:57-70.

57. Rozakis-Adock M FR, Wade J, Pawson T, Bowtell D. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 1993;363:83-85.

58. J D. The Grb2/Sem-5 Adaptor Protein. FEBS Lett 1994;338:113-117.

59. Di Fulvio M, Henkels KM, Gomez-Cambronero J. Short-hairpin RNA-mediated stable silencing of Grb2 impairs cell growth and DNA synthesis. Biochem Biophys Res Commun 2007;357(3):737-42.

60. Yu GZ, Chen Y, Long YQ, Dong D, Mu XL, Wang JJ. New insight into the key proteins and pathways involved in the metastasis of colorectal carcinoma. Oncol Rep 2008;19(5):1191-204.

61. Di Fulvio M, Frondorf K, Henkels KM, Lehman N, Gomez-Cambronero J. The Grb2/PLD2 interaction is essential for lipase activity, intracellular localization and signaling in response to EGF. J Mol Biol 2007;367(3):814-24.

62. Schlessinger J. SH2/SH3 signaling proteins. Curr Opin Genet Dev 1994;4(1):25-30.

63. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 1993;363(6424):45-51.

64. Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 1992;70(3):431-42.

65. C. Zhao GD, K. Showronek, M.A. Frohman and D. Bar-Sagi. Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. Nat Cell Biol 2007;9:706-712.

66. Henkels KM, Short S, Peng HJ, Di Fulvio M, Gomez-Cambronero J. PLD2 has both enzymatic and cell proliferation-inducing capabilities, that are differentially regulated by phosphorylation and dephosphorylation. Biochem Biophys Res Commun 2009;389(2):224-8.

67. Chae YC, Kim JH, Kim KL, Kim HW, Lee HY, Heo WD, Meyer T, Suh PG, Ryu SH. Phospholipase D activity regulates integrin-mediated cell spreading and migration by inducing GTP-Rac translocation to the plasma membrane. Mol Biol Cell 2008;19(7):3111-23.

68. Bokoch GM. Regulation of innate immunity by Rho GTPases. Trends Cell Biol 2005;15(3):163-71.

69. Ambruso DR, Knall C, Abell AN, Panepinto J, Kurkchubasche A, Thurman G, Gonzalez-Aller C, Hiester A, deBoer M, Harbeck RJ, Oyer R, Johnson GL, Roos D. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. Proc Natl Acad Sci U S A 2000;97(9):4654-9.

70. Wang L, Zheng Y. Cell type-specific functions of Rho GTPases revealed by gene targeting in mice. Trends Cell Biol 2007;17(2):58-64.

71. Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, Kwiatkowski DJ, Williams DA. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science 2003;302(5644):445-9.

72. Pan Y, Bi F, Liu N, Xue Y, Yao X, Zheng Y, Fan D. Expression of seven main Rho family members in gastric carcinoma. Biochem Biophys Res Commun 2004;315(3):686-91.

73. Abraham MT, Kuriakose MA, Sacks PG, Yee H, Chiriboga L, Bearer EL, Delacure MD. Motility-related proteins as markers for head and neck squamous cell cancer. Laryngoscope 2001;111(7):1285-9.

74. Stephen J. Kempiak S-CY, Jonathon M. Backer, and Jefferey E. Segall. Local signaling by the EGF receptor. J Cell Biology 2003;162:781-787.

75. Lurje G LH. EGFR signaling and drug discovery. Oncology 2009;77:400-410.

76. Burgess AW CH, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, Lemmon MA, Sliwkowski MX, Ward CW, Yokoyama S. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell 2003;12:541-552.

77. Dawson JP BM, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. Mol Cell Biol 2005;25(7734-7742).

78. Klein P MD, Lemmon MA, Schlessinger J. A structure-based model for ligand binding and dimerization of EGF receptors. Proc Natl Acad Sci U S A 2004;101:929-934.

79. Balvinder S. Shoker CJ, Robert B. Clarke, Elizabeth Anderson, Joanne Hewlett, Michael P.A. Davies, D. Ross Sibson, and John P. Sloane. Estrogen Receptor-Positive Proliferating Cells in the Normal and Precancerous Breast. Am J Pathol 1999;155:1811-1815.

80. Joshua Chuck Harrell WWD, Djuana M.E. Harvell, Mauricio Pinto, Paul Jedlicka, Carol A. Sartorius and Kathryn B. Horwitz. Estrogen Insensitivity in a Model of Estrogen-Receptor Positive Breast Cancer Lymph Node Metastasis. Cancer Res 2007;67:10582.

81. Radisky DC. Epithelial-mesenchymal transition. J Cell Sci 2005;118:4325-4326.

82. Higgins RSaPJ. MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitior type 1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. J Cell Sci 2002;115:3093-3103.

83. Weigang Wang JBW, Victoria Centonze Forhlich, Yuri Oleynikov, Stefan Huttelmaier, Jiri Zavadil, Lukas Cermak, Erwin P. Bottinger, Robert H. Singer, John G. White, Jeffrey E. Segall and John S. Condeelis. Single Cell Behavior in Metastatic Primary Mammary Tumors Correlated with Gene Expression Patterns Revealed by Molecular Profiling. Cancer Res 2002;62:6278-6288.

84. Su W YO, Olepu S, Genna A, Park JS, Ren H, Du H, Gelb MH, Morris AJ, and Michael A. Frohman. 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a Phospholipase D Pharmalogical Inhibitor That Alters Cell Spreading and Inhibits Chemotaxis. Mol Pharm 2009;75:437-446.

85. Patel D SD, Gupta S. Apigenin and cancer chemoprevention: Progress, potential and promise (Review). International Journal of Oncology 2007;30:233-245.

86. JK LSaL. Inhibitory effects of phytopolyphenols on
TPA-induced transformation, PKC activation, and c-jun expression in mouse fibroblast cells. Nutr Cancer 1997;28:177-183.

87. Lin JK CY, Huang YT and Lin-Shiau SY. Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin
and curcumin. J Cell Biochem 1997;28(39-48).

88. BD MBaT. The extracellular signal-regulated kinase pathway contributes to mitogenic and anti-apoptotic effects of peroxisome proliferators in vitro. Toxicol Appl Pharmacol 1999;159:125-133.

89. Yin F GAavHA. Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells (ARO). Anticancer Res 1999;19:4297-4303.

90. Chardin P CJ, Gale NW, van Aelst L, Schlessinger J, Wigler MH, Bar-Sagi D. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to Grb2. Science 1993;260:1338-1343.

91. Gomez-Cambronero J, Di Fulvio M, Knapek K. Understanding phospholipase D (PLD) using leukocytes: PLD involvement in cell adhesion and chemotaxis. J Leukoc Biol 2007;82(2):272-81.

92. Ho CC LK, Hsu SC, Kuo CL, Ma CY, Lin ML, Yang JS, Chung JS. Benzyl isothiocyanate (BITC) inhibits migration and invasion of human gastric cancer AGS cells via suppressing ERK signal pathways. Hum Exp Toxicol 2010.

93. Parker LP TD, Kesterson S, Gercel-Taylor C. Gene expression profiling in response to estradiol and genistein in ovarian cancer cells. Cancer Genomics Proteomics 2009;6:189-194.

94. Lu Z, Hornia A, Joseph T, Sukezane T, Frankel P, Zhong M, Bychenok S, Xu L, Feig LA, Foster DA. Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts. Mol Cell Biol 2000;20(2):462-7.

95. Joseph T, Wooden R, Bryant A, Zhong M, Lu Z, Foster DA. Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2. Biochem Biophys Res Commun 2001;289(5):1019-24.

96. Huang Y QI, Chen H. Effects of phosphatidylinositol 4,5-bisphosphate and neomycin on phosplipase D: kinetic studies. Mol Cell Biochem 1999;197:195-201.

97. Vitale N CA, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA, Bader MF. Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. Embo J 2001;20:2424-2434.

98. Garcia A ZY, Zhao C, Toschi A, Fan J, Shraibman N, Brown HA, Bar-Sagi D, Foster DA, Arbiser JL. Honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in human cancer cells. Clin Cancer Res 2008;14:4267-4274.